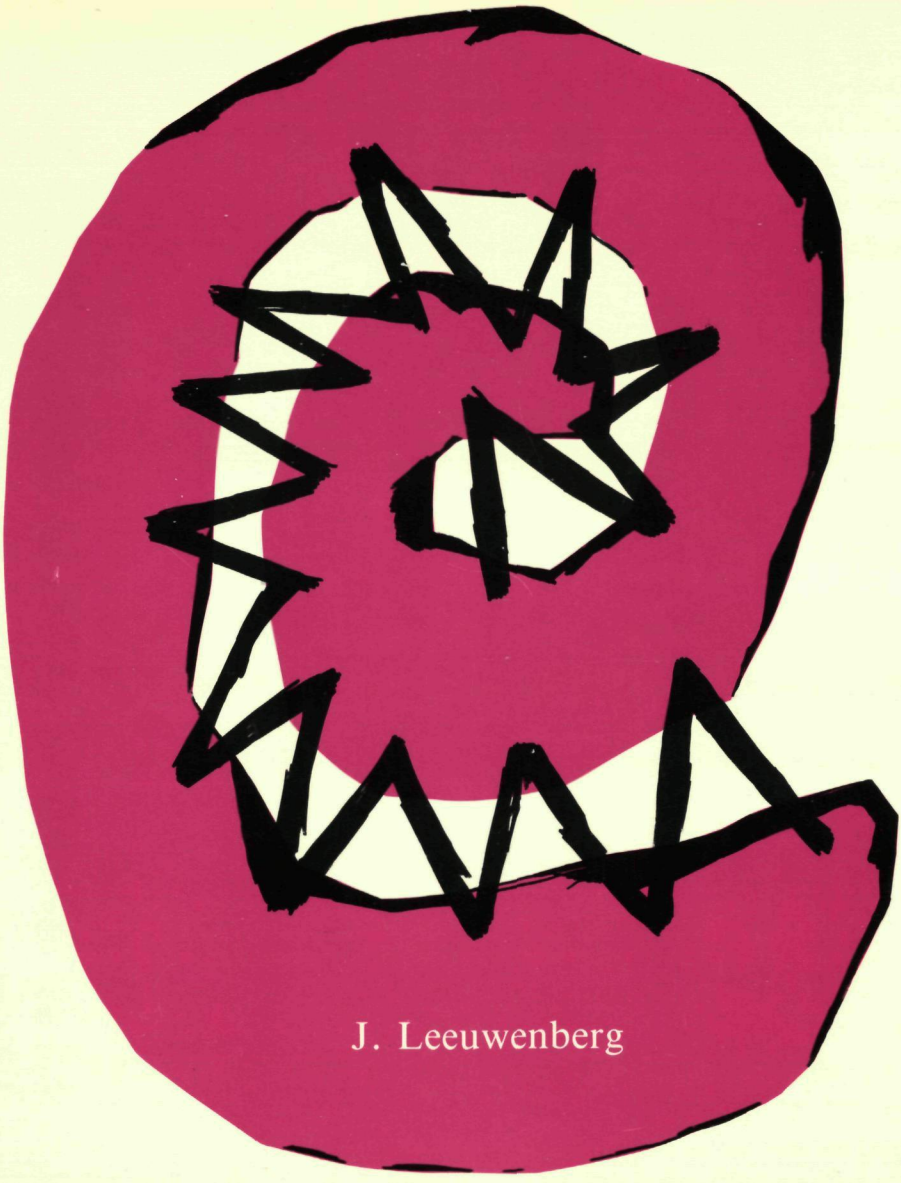

J. Leeuwenberg

# Functional studies on monoclonal antibodies directed against the T cell differentiation antigens CD3 and CD7

# Functional studies on monoclonal antibodies directed against the T cell differentiation antigens CD3 and CD7

# Functional studies on monoclonal antibodies directed against the T cell differentiation antigens CD3 and CD7

een wetenschappelijke proeve
op het gebied van de geneeskunde
en tandheelkunde

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit te Nijmegen,
volgens besluit van het college van decanen
in het openbaar te verdedigen
op vrijdag 15 april 1988
des namiddags te 1.30 uur precies

door

Henriëtte Frederica Maria Leeuwenberg

geboren op 18 mei 1953 te Heerlen

Drukkerij Groenevelt b.v. – Landgraaf

Promotores : Prof. Dr. P.J.A. Capel (R.U. Utrecht)
Prof. Dr. R.A.P. Koene

Coreferent : Dr. W.J.M. Tax

This thesis was prepared at the Department of Medicine, Division of Nephrology (Head Prof. Dr. R.A.P. Koene),
St. Radboud Hospital, University of Nijmegen, Nijmegen, The Netherlands.
I want to express my gratitude to all, who have contributed to the completion of this thesis.

*Aan mijn ouders*

Cover design: Miriam van Velthoven

Financial support by The Dutch Kidney Foundation for the publication of this thesis is gratefully acknowledged.

CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| ALG | anti-lymphocyte globulin |
| ATG | anti-thymocyte globulin |
| CD | cluster of differentiation |
| CML | cell-mediated lympholysis |
| CTL | cytotoxic T lymphocyte |
| FcR | Fc-receptor |
| HS | human serum |
| Ig | immunoglobulin |
| IL | interleukin |
| kD | kilo Dalton |
| LFA | lymphocyte function associated antigen |
| MAb | monoclonal antibody |
| MHC | major histocompatibility complex |
| PBL | peripheral blood lymphocytes |
| T-ALL | thymic acute lymphoblastic leukemia |
| TCR | T cell receptor |

# CHAPTER I

# INTRODUCTION

Immunological research has made enormous progress during the last ten years, thanks to the development of new techniques, such as the cloning of T cells, the recombinant DNA technique, and the production of hybridomas. T cell clones are used to study cellular interactions, production of lymphokines, and the recognition of antigen. The application of the recombinant DNA technique has given information about gene structures, including MHC genes, oncogenes and oncogene-related gene products, and the recombination during the rearrangements of genes in B and T cell ontogeny. The hybridoma technique, which was introduced in 1975 by Köhler and Milstein (1), implies the immortalization of antibody-producing cells (B cells). Immortalization of the cells is established by fusion of spleen cells, harvested after appropriate immunization, with continuously growing mouse myeloma cells. These hybrid cells can be cultured and cloned in vitro, and have the combined properties of the parental cells; the capacity of the spleen B cell to produce antibodies with a distinct specificity and the continuous growth potential of the myeloma cell. In this way reagents can be obtained in large quantities that are highly specific for the antigen which was used for immunization. Originally, the technique was developed to investigate the nature and genetic origin of antibody diversity, using monoclonal antibodies (MAb) directed against sheep red blood cells. The potential value of this technique as an immunological tool in diagnostic, therapeutic, and analytical research was soon realized. Up till now, a large number of MAb have been described against viral, bacterial, parasitic, and tumour antigens, against haptens, hormones, and cell surface antigens.

**T cell differentiation antigens.**

MAb directed against cell surface antigens have been used as an instrument to study the morphology and morphogenesis of tissues. Especially the study of the differentiation of lymphoid cells has been developed thanks to the monoclonal antibody technique. Lymphoid cells are derived from the bone marrow, where stem cells reside. The progeny of pluripotent or committed stem cells migrate from the bone marrow into the peripheral lymphoid organs to differentiate into mature T or B lymphocytes. The T cell precursor cells migrate as prothymocytes into the thymus, where further maturation occurs. In 1980 Reinherz et al. (2) developed a hypothetical scheme of the origin of T cells as analyzed with MAb. This scheme has been supplemented later by several

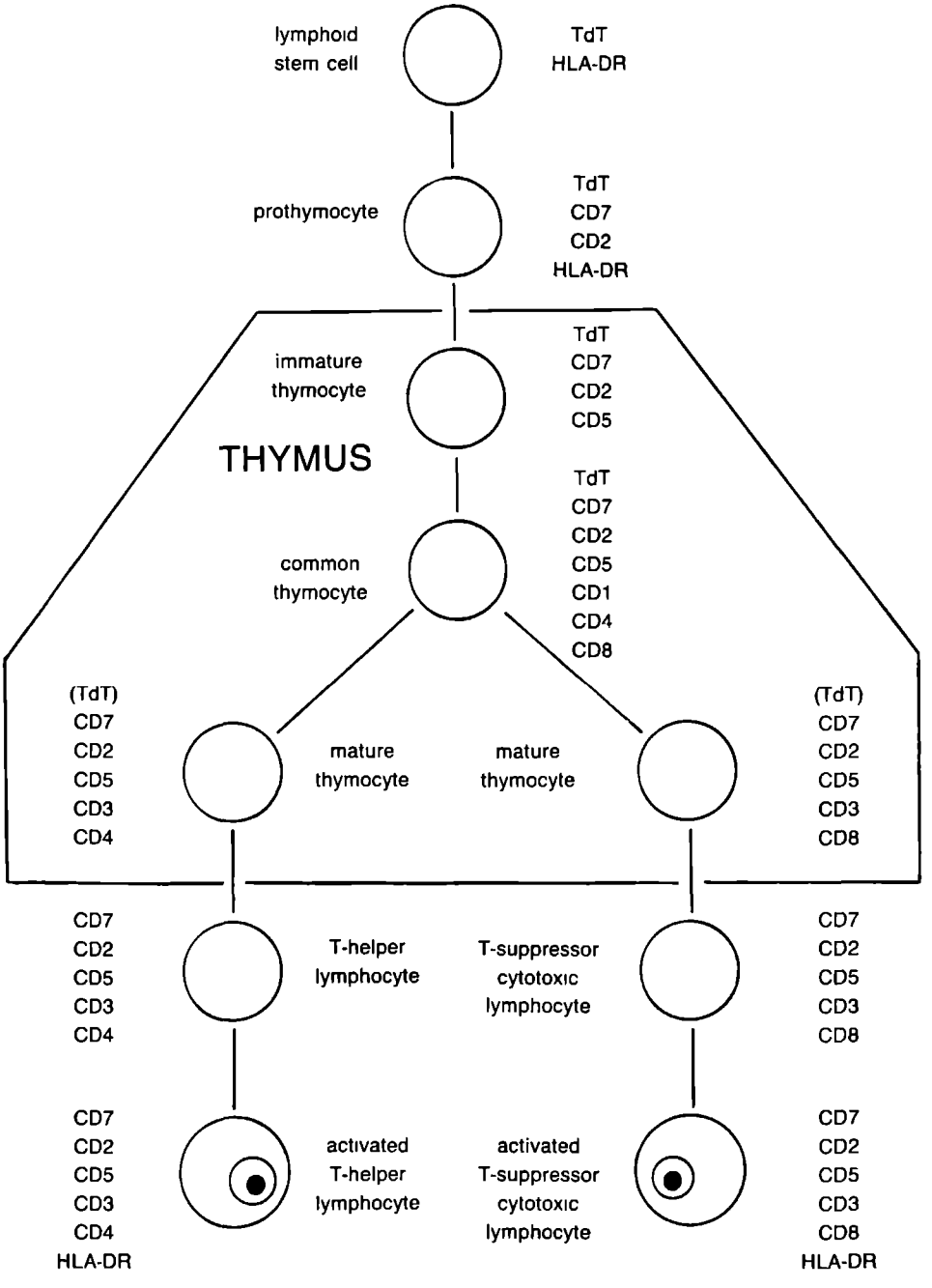


*Figure 1. T cell differentiation.*

investigators (fig.1). Since the nomenclature of the T cell differentiation antigens varied per research group, a universal nomenclature was proposed during the First International Workshop on Human Leukocyte Differentiation Antigens to avoid difficulties in communication. At present seven Clusters of Differentiation (CD) on T cells or T cell subsets have been defined.

The lymphoid stem cell is characterized by the presence of HLA-DR and by the nuclear enzyme terminal transferase (TdT). Although its function is not clear, it has been suggested that TdT is involved in increasing diversity in the D (diversity) J (joining) segments of receptor genes (3). The prothymocyte expresses additionally CD7 (a 40 kD glycoprotein) (4), and CD2. The CD2 molecule, also named T11, was originally described as the sheep red blood cell receptor, and is a 50-58 kD glycoprotein (5). It binds to the cell surface antigen LFA-3, and can mediate adhesion of lymphoid cells via interaction with LFA-3 (6). The immature common thymocyte acquires subsequently the CD5 (67 kD) (7), CD1 (40 kD) (8), CD4 (55 kD) (2) and CD8 (a heterodimer of 76 kD) (2) antigens. Upon further maturation CD1 and either CD4 or CD8 are lost from the cell surface (2). The mature thymocytes are immunocompetent cells and express the CD3 antigen. The CD3 antigen consists of a group of noncovalently associated proteins: $-\gamma$ (25 kD), $-\delta$ (20 kD), $-\epsilon$ (20 kD), and $-\zeta$ (16 kD) chain (9, 10). This antigen complex is closely associated with the T cell receptor (TCR), a disulphide-linked variable heterodimer of 90 kD ($\alpha$ and $\beta$ chain), or, on a small minority of the peripheral T cells (less than 5%), with the T cell receptor $\gamma$ (TCR$\gamma$) polypeptide (11). The CD3 positive cells enter the peripheral blood as antigen-specific T lymphocytes, being either CD4 or CD8 positive. The CD4 positive cells (approximately 65% of the peripheral T cells) are generally MHC-class II restricted in the recognition of antigen, and are mainly involved in helper and inducer functions. The CD8 positive cells (approximately 35% of the peripheral T cells) include the suppressor/cytotoxic T cells, and are mainly MHC-class I restricted in the recognition of antigen. The CD4 and CD8 markers do not provide a strict separation of these different functional properties, since CD4 positive cytotoxic T cell clones have been described (12, 13). Similarly, CD4 positive MHC-class I specific, and CD8 positive MHC-class II specific cells have been found (12, 14, 15). The production of interleukin-2 (IL2), the essential T cell growth factor necessary for growth of T cells, natural killer (NK) cells and of B cells (16), is a property of CD4 positive cells as well as CD8 positive cells (17).

**Functional aspects of T cell differentiation antigens.**

The use of MAb directed against the different antigens in functional assays, has provided insight into the function of the T cell antigens. For example, the CD4, CD8, CD2, and the CD3 antigens are each in their own way involved in the recognition of antigen. The recognition of antigen by CD4 positive cells or CD8 positive cells can be blocked by MAb anti-CD4 or anti-CD8 respectively, as revealed by inhibition studies of the antigen-specific cytotoxicity. Anti-CD4 MAb block the MHC-class II restricted lysis by CD4-positive cytotoxic T lymphocytes (CTL), whereas anti-CD8 MAb inhibit the MHC-class I restricted lysis by CD8-positive CTL, both at the adhesion step in the cytolytic process. Therefore, the usual ligands for CD4 and CD8 are thought to be MHC-class II and class I molecules respectively. The putative function of these molecules is to stabilize the conjugate formation between the target cell and the effector cell. Recently, however, it has been described, that anti-CD4 MAb could also block the proliferation response of CD4 positive lymphocytes induced by mitogenic stimuli. This inhibition occurs at a late stage in the T cell activation process, since the activation pulse, as measured by the rise of cytoplasmic $Ca^{2+}$, is not affected. Therefore, the CD4 molecule is thought to be involved in a post-activation event of CD4-positive lymphocytes (18). A similar role has been suggested for the CD8 molecule, since anti-CD8 MAb could inhibit the lytic activity of CTL after the binding of CTL to the target cell. It has been suggested, that the CD8 molecule plays a role in negative signalling to the T cell, preventing T cell triggering by nonspecific interaction (19). The CD2 antigen has also a function in the interaction of the T cells with antigen, since some, but not all, MAb directed against CD2 are able to block antigen dependent helper T cell proliferation and to inhibit the cytolytic process at the step of the conjugate formation (20). It has been proposed, that distinct epitopes on CD2 are involved in different processes, since MAb directed against different epitopes on CD2 can have different effects in functional assays (21). The CD2 antigen appears to be involved in T cell activation, since a combination of two MAb, directed against different epitopes on CD2 termed $T11_2$ and $T11_3$, are able to induce T cell proliferation and IL2 release (22). Triggering of the CD2 antigen by anti-CD2 MAb leads to an increase of intracellular free $Ca^{2+}$ (23). Moreover, it has been described that the combination of anti-$T11_2$ and anti-$T11_3$ MAb induce an antigen nonspecific cytolytic activity in cytolytic T cells (24).
Anti-CD3 MAb can have apparently opposite effects on T cells. On the

one hand, they block T cell activities, such as the proliferation of T cells to soluble antigen (25), or the antigen specific cytotoxicity (26). On the other hand anti-CD3 MAb induce activation of T cells, e.g. an increase of intracellular free $Ca^{2+}$, mitogenesis, expression of IL2-receptors, secretion of gamma-interferon by T cells (23, 27, 28, 29), and induce a nonspecific lytic activity by cytotoxic T cells (this thesis). Thus, anti-CD3 MAb either block antigen specific responses, or mimic the activation process, depending on the assay used. This reactivity pattern can be explained by the finding, that the CD3 antigen is associated with the TCR. The TCR serves for the specific recognition of antigen, whereas subsequently the CD3 complex transduces the signal through the cell membrane.
Other T cell differentiation antigens are the CD7 molecule and the CD5 molecule. Anti-CD7 MAb have no effects on T lymphocytes in in vitro assays, but have immunosuppressive activities in a skin allograft model, suggesting that this antigen is important for T cell function (this thesis). Anti-CD5 MAb can bypass the need for accessory cells in T cell activation, providing the necessary additional signal (30).

**Application of MAb directed against T cell differentiation antigens.**

MAb directed against lymphocyte surface antigens have already been used in the diagnosis and treatment of many diseases. For a number of diseases, an association has been established between increased or decreased number of suppressor cells, as judged by CD4/CD8 ratios and disease activity (31). Also for the diagnosis of lymphomas and leukemias, antibodies directed against lymphocyte differentiation antigens have proved to be highly useful. One of the antibodies discussed in this thesis WT1 (anti-CD7) is very useful in the diagnosis of T-ALL (thymic acute lymphoblastic leukemia) (32) and proved to be a good candidate for immunotherapy, when conjugated to the toxin Ricin A (33). Besides their diagnostic value, in vivo therapy with MAb is a rapidly developing field and one of the applications is the induction of immunosuppression. Already 20 years ago polyclonal antibodies directed against lymphocytes (ALG, anti-lymphocyte globulin) have been used as an immunosuppressive drug in clinical transplantation (34). The first randomized clinical trial where more specific antiserum, i.e. anti-thymocyte globulin (ATG), was used to reverse renal allograft rejection, was undertaken in 1979. ATG appeared to be very effective in improving graft survival (35).

**MAb for immunosuppressive therapy.**

To further increase the specificity of antibody treatment by removing defined lymphoid populations, and to reduce the amount of foreign protein administered to the patients, MAb directed against T cells have been tested. An obvious choice is anti-CD3 MAb, since this antibody is able to block the proliferative response to antigen in vitro and the cytotoxic effector function of the T cells. OKT3 (anti-CD3) MAb has been successfully used to treat acute renal allograft rejection (36, 37). Another anti-CD3 MAb WT32 proved also to be suitable for rejection treatment (38). Many other monoclonals have been applied for this purpose, but they all proved to be less powerful than anti-CD3. WT1 (anti-CD7), tested in the Rhesus monkey (this thesis) and OKT4 (anti-CD4), tested in the Cynomolgus monkey (39) were shown to improve the survival of skin and renal allografts respectively.
The mechanism by which anti-CD3 MAb exerts its function as an immunosuppressive drug is not yet clear and needs further investigation. Probably, T cells are eliminated via the mononuclear phagocytic system following opsonization. Binding of anti-CD3 MAb also causes modulation and internalization of the CD3-antigen. In this way the T cells loose the CD3-antigen and are rendered nonreactive to antigen.

**Fc-receptors and immunosuppressive therapy.**

The requirement for Fc-mediated processes in the induction of immunosuppression has been demonstrated by the finding that F(ab')2 fragments of polyclonal ATG were ineffective (40). In many applications of MAb in vivo, Fc-mediated mechanisms are triggered which can be beneficial, e.g. elimination of target cells, activation of effector cells, elimination of soluble antigen. Therefore, it is of importance to investigate the interaction of murine antibodies with the receptors for IgG on human cells.
The interaction of the Fc-receptor with IgG is more complicated and heterogeneous than was thought hitherto. Fc-receptors on macrophages are involved in endocytosis of immune complexes (41), antibody dependent cell-mediated cytotoxicity (ADCC) (42), stimulation of the release of inflammatory mediators (43), and superoxide production (44). Analysis of the murine Fc-receptor revealed that individual cells may have more than one type of Fc-receptor; mouse macrophages possess three types of Fc-receptors for murine IgG; a trypsin sensitive receptor for monomeric IgG2a (FcRI) (45); a trypsin

resistent receptor, binding preferentially aggregated IgG1 and IgG2b antibodies (FcRII) (46); and a receptor for aggregated IgG3 (FcRIII) (47). Heterogeneity in specificity of Fc-receptors is found for different cell types: mouse B cells carry an Fc-receptor, binding murine IgG1, IgG2a, and IgG2b, but not IgG3, which thus differs in its specificity from the Fc-receptors on macrophages (48).
In man, three types of Fc-receptors for human IgG are known; 1) a 72 kD (FcRI), 2) a 40 kD Fc-receptor (FcRII), and 3) an Fc-receptor with low affinity (50-70 kD) ($FcR_{lo}$) (49, 50, 51). The human FcRI (HFcRI) is present on monocytes, whereas the HFcRII is also present on macrophages, platelets, granulocytes and B cells. The $HFcR_{lo}$ is expressed on neutrophils, eosinophils, macrophages, natural killer cells, killer cells, large granular lymphocytes, and $T_\gamma$ cells (52). Some homology with murine Fc-receptors was supposed, since HFcRI binds selectively murine IgG2a, and HFcRII interacts with murine IgG1 (49). Sofar, however, the homologue of the $HFcR_{lo}$ has not been found on mouse cells.

**Aim of this study**

The aim of this study was to investigate some biological activities of the pan-T cell differentiation antigens CD3 and CD7. The functional studies were initiated in view of possible future application of MAb for clinical immunosuppression. The CD7 antigen was chosen, because its expression strongly increases upon activation of T cells. In Chapter II a MAb (WT1) is characterized and tested for its immunosuppressive actitvity in a Rhesus monkey model. Its value for diagnosis of T-ALL and its therapeutic use are discussed.
In Chapter III a novel effect of MAb directed against the CD3/TCR complex is described: the induction of nonspecific cytotoxicity, i.e. antigen specific cytotoxic T-cell lines as well as cytotoxic T-cell clones (Chapter IV) can be triggered by anti-CD3 MAb to mediate antigen-nonspecific cytotoxic activity. This process is Fc-dependent; crosslinking occurs between the Fc-part of the anti-CD3 MAb and the FcR on the target membrane, resulting in an effective triggering of the cytotoxic T cell. Not only FcR-positive cells, but also CD3 positive cells are prone to this induced lysis (Chapter V).
Knowledge regarding human FcR for murine antibodies is of great importance, since murine MAb will be widely used in vivo for therapeutical application, e.g. antitumor therapy and anti-rejection therapy. Because the antibodies exert their function in many processes via FcR, the study of the interaction of murine antibodies with human

FcR is of great importance. The anti-CD3 induced cytotoxicity appeared to be a useful tool to provide information on the interaction of FcR on human cells with murine IgG. In Chapter VI a study on the characteristics and functional properties of FcR for murine IgG on human monocytes and B cells is described.

REFERENCES


1. Kohler, G., Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256:495-497.
2. Reinherz, E., Schlossman, S. The differentiation and function of human T lymphocytes. Cell 1980; 19:821-827.
3. Kunkel, T., Gopinathan, K., Dube, D., Snow, E., Loeb, L. Rearrangements of DNA mediated by terminal transferase. Proc. Natl. Acad. Sci. USA 1986; 83:1867-1871.
4. Link, M., Warnke, R., Finlay, J., Amylon, M., Miller, R., Dilley, J., Levy, R. A single monoclonal antibody identifies T-cell lineage of childhood lymphoid malignancies. Blood 1983; 62:722-728.
5. Howard, F., Ledbetter, J., Wong, J., Bieber, C., Stinson, E., Herzenberg, L. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. J. Immunol. 1981; 126:2117-2122.
6. Selvarej, P., Plunkett, M., Dustin, M., Sanders, M., Shaw, S., Springer, T. The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. Nature 1987; 326:400-403.
7. Engleman, E., Warnke, R., Fox, R., Levy, R. Studies of a human T lymphocyte antigen recognized by a monoclonal antibody. Proc. Natl. Acad. Sci. USA 1981; 78:1791-1795.
8. Bhan, A., Reinherz, E., Poppema, S., McCluskey, R., Schlossman, S. Location of T cell and major histocompatibility complex antigens in the human thymus. J. Exp. Med. 1980; 152:771-782.
9. Borst, J., Alexander, S., Elder, J., Terhorst, C. The T3 complex on human T lymphocytes involves four structurally distinct glycoproteins. J. Biol. Chem. 1983; 258:5135-5141.
10. Weissman, A., Samelson, L., Klausner, R. A new subunit of the human T-cell antigen receptor complex. Nature 1986; 324:480-482.
11. Borst, J., Griend, R. van de, Oostveen, W. van, Ang, S., Melief, C., Seidman, J., Bolhuis, R. A T-cell receptor Y/CD3 complex found on cloned functional lymphocytes. Nature 1987; 325:683-688.
12. Spits, H., Yssel, H., Thompson, A., De Vries, J. Human T4+ and T8+ cytotoxic T lymphocyte clones directed at products of different class II major histocompatibility complex loci. J. Immunol. 1983; 131:678-683.
13. Krensky, A., Reiss, C., Mier, J., Strominger, J., Burakoff, S. Longterm human cytolytic T-cell lines allospecific for HLA-DR6 antigen are OKT4+. Proc. Natl. Acad. Sci. USA 1982; 79:2365-2369.
14. Flomenberg, M., Duffy, E., Naito, K., Dupont, B. Two distinct phenotypes of HLA-DR-specific cytotoxic T-cell lines. Immunogenetics 1983; 17:317-324.
15. Flomenberg, N., Naito, K., Duffy, E., Knowles, R., Evans, R., Dupont, B. Allocytotoxic T cell clones. Both Leu 2+,3- and Leu2-,3+ T cells recognize class I histocompatibility antigens. Eur. J. Immunol. 1983; 13:905-911.
16. Tsudo, M., Uchiyama, T., Uchino, H. Expression of Tac antigen on activated normal human B cells. J. Exp. Med. 1984; 160:612-617.
17. Luger, T., Smolen, J., Chused, T., Steinberg, A., Oppenheim, J. Human lymphocytes with either the OKT4 or OKT8 phenotype produce interleukin 2 in culture. J. Clin. Invest. 1982; 70:470-473.
18. Carrera, A., Sanchez-Madrid, F., Lopez-Botet, M., Bernabeu, C., De Landazuri, M. Involvement of the CD4 molecule in a post-activation event on T cell proliferation. Eur. J. Immunol. 1987; 17:179-186.
19. Seventer, G. van, Lier, R. van, Spits, H., Ivanyi, P., Melief, C. Evidence for a regulatory role of the T8 (CD8) antigen in antigenspecific and

anti-T3(CD3)-induced lytic activity of allospecific cytotoxic T lymphocyte clones. Eur. J. Immunol. 1987; 16:1363-1371.
20. Krensky, A., Robbins, E., Springer, T., Burakoff, S. LFA-1, LFA-2, and LFA-3 antigens are involved in CTL-target cell conjugation. J. Immunol. 1984; 132:2180-2183.
21. Bolhuis, R., Gravekamp, C., Griend, R. van de. Cell-cell interactions. Clin. Immunol. Allergy 1986; 6:28-90.
22. Meuer, S., Hussey, R., Fabbi, M., Fox, D., Acuto, O., Fitzgerald, K., Hodgdon, J., Protentis, J., Schlossman, S., Reinherz, E. An alternative pathway of T-cell activation: a functional role for the 50 KD T11 sheep erythrocyte receptor protein. Cell 1984; 36:897-906.
23. Weiss, J., Daley, J., Hodgdon, J., Reinherz, E. Calcium dependency of antigen-specific (T3-Ti) and alternative (T11) pathways of human T-cell activation. Proc. Natl. Acad. Sci. USA 1984; 81:6836-6840.
24. Siliciano, R., Pratt, J., Schmidt, R., Ritz, J., Reinherz, E. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature 1985; 317:428-430.
25. Meuer, S., Fitzgerald, K., Hussey, R., Hodgdon, J., Schlossman, S. Reinherz, E. Clonotypic structures involved in antigen specific human T cell function: relationship to the T3 molecular complex. J. Exp. Med. 1983; 157:705-719.
26. Chang, T., Kung, P., Gingras, S., Goldstein, G. Does OKT3 monoclonal antibody react with an antigen-recognition structure on human T cells? Proc. Natl. Acad. Sci. USA 1981; 78:1805-1808.
27. Wauwe, J. van, de Mey, J., Goossens. J. OKT3: a monoclonal antihuman T lymphocyte antibody with potent mitogenic properties. J. Immunol. 1980; 124:2708-2713.
28. Tsoukas, C., Landgraf, B., Bentin, J., Valentine, M., Lotz, M., Vaughan, J., Carson, D. Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes. J. Immunol. 1985; 135:1719-1723.
29. Von Wussow, P., Platsoucas, C., Wiranowska-Stewart, M., Stewart, W. Human γ interferon production by leukocytes induced with monoclonal antibodies recognizing T cells. J. Immunol. 1981; 127:1197-1200.
30. Ceuppens, J., Baroja, M. Monoclonal antibodies to the CD5 antigen can provide the necessary second signal for activation of isolated resting T cells by solid-phase-bound OKT3. J. Immunol. 1986; 137:1816-1821.
31. Krensky, A., Clayberger, C. Diagnostic and therapeutic implications of T cell surface antigens. Transplantation 1985; 39:339-348.
32. Vodinelich, L., Tax, W., Bai, Y., Pegram, S., Capel, P., Greaves, M. A monoclonal antibody (WT1) for detecting leukemias of T-cell precursors (T-ALL). Blood 1983; 62:1108-1113.
33. Myers, C., Thorpe, P., Ross, W., Cumber, A., Katz, F., Tax, W., Greaves, M. An immunotoxin with therapeutic potential in T cell leukemia: WT1-ricin A. Blood 1984; 63:1178-1185.
34. Starzl, T., Marchioro, T., Porter, K., Iwasaki, Y., Cerilli, G. The use of heterologous antilymphoid agents in canine renal and liver homotransplantation and in human renal homotransplantation. Surg. Gynecol. Obstet. 1967; 124:301-318.
35. Shield, C., Cosimi, A., Tolkoff-Rubin, N., Rubin, R., Herrin, J., Russel, P. Use of antithymocyte globulin for reversal of acute allograft rejection. Transplantation 1979; 28:461-464.
36. Ortho Multicenter Transplant Study group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. N. Engl. J. Med. 313:337.
37. Cosimi, A., Cho, S., Delmonico, F., Kaplan, M., Rohrer, R., Jenkins, R. A

randomized clinical trial comparing OKT3 and steroids for treatment of hepatic allograft rejection. Transplantation 1987; 43:91-95.
38. Tax, W., van de Heijden, H., Willems, H., Hoitsma, A., Berden, J., Capel, P., Koene, R. Immunosuppression with monoclonal anti-T3 antibody (WT32) in renal transplantation. Transplant. Proc. 1987; 19:1905-1907.
39. Cosimi, A., Burton, R., Kung, P., Colvin, R., Goldstein, G., Lifter, J., Rhodes, W., Russell, P. Evaluation in primate renal allograft recipients of monoclonal antibody to human T-cell subclasses. Transplant. Proc. 1981; 13:499-503.
40. Lance, E., Medawar, P., Taub, R. Antilymphocyte Adv. Immunol. 1973; 17:1-92.
41. Silverstein, S., Steinman, R., Cohn, Z. Endocytosis. Ann. Rev. Biochem. 1977; 46:669-722.
42. Cerottini, J., Brunner, K. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. Adv. Immunol. 1974; 18:67-132.
43. Rouzer, C., Scott, W., Kempe, W., Cohn, Z. Prostaglandin synthesis by macrophages requires a specific receptor-ligand interaction. Proc. Natl. Acad. Sci. USA 1980; 77:4279-4282.
44. Yamamoto, K., Johnson, R. Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages. J. Exp. Med. 1984; 159:405-416.
45. Unkeless, J., Eisen, H. Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. J. Exp. Med. 1975; 142:1520-1533.
46. Heusser, C., Anderson, C., Grey, H. Receptors for IgG: subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. J. Exp. Med. 1977; 145:1316-1327.
47. Diamond, B., Yelton, D. A new Fc receptor on mouse macrophages binding IgG3. J. Exp. Med. 153:514-519.
48. Teillaud, J., Diamond, B., Pollock, R., Fajtova, V., Scharff, M. Fc receptors on cultured myeloma and hybridoma cells. J. Immunol. 1985; 134:1774-1779.
49. Kurlander, R., Batker, J. The binding of human IgG1 monomer and small, covalently cross-linked polymers of IgG1 to human peripheral blood monocytes and polymorphonuclear leukocytes. J. Clin. Invest. 1982; 69:1-8.
50. Looney, J., Abraham, G., Anderson, C. Human monocytes and U937 cells bear two distinct Fc receptors for IgG. J. Immunol. 1986; 136:1641-1647.
51. Kulczycki, A. Human neutrophils and eosinophils have structurally distinct $Fc_\gamma$ receptors. J. Immunol. 1984; 133:849-854.
52. Anderson, C., Looney, R. Human leukocyte IgG Fc receptors. Immunol. Today 1986; 7:264-266.

# CHAPTER II

**Monoclonal antibody (WT1) directed against a T cell surface glycoprotein: characteristics and immunosuppressive activity.**

Clin. exp. Immunol. 1984. 55:427

Tax, W., N. Tidman, G. Janossy, L. Trejdosiewicz, H. Willems, J. Leeuwenberg, T. De Witte, P. Capel, R. Koene.

*Clin exp Immunol* (1984) **55**, 427–436

# Monoclonal antibody (WT 1) directed against a T cell surface glycoprotein: characteristics and immunosuppressive activity

W J M TAX, N TIDMAN,* G JANOSSY,* L TREJDOSIEWICZ,* RIKI WILLEMS, JET LEEUWENBERG, T J M DE WITTE, P J A CAPEL & R A P KOENE *Departments of Nephrology, Pathology and Haematology, St Radboudziekenhuis, Nijmegen, The Netherlands and * Department of Immunology, Royal Free Hospital School of Medicine London UK*

*(Accepted for publication 19 September 1983)*

## SUMMARY

WT 1, an IgG2a subclass monoclonal antibody, recognizes a human T lineage specific antigen (mol wt 40,000). This antigen is strongly expressed on thymic T blasts, and on peripheral T cells activated by phytohaemagglutinin, whereas cortical thymocytes and peripheral T cells are moderately positive for WT 1. In contrast, B lymphocytes, myeloid and erythroid cells, including the progenitor cells of these lineages, and terminal deoxynucleotidyl transferase positive cells in the bone marrow, are all WT 1 negative. Binding of WT 1 to T cells is blocked by a previously described antibody (3A1) suggesting that both antibodies bind to the same antigen present on human T cells. WT 1, however, is also reactive with T lymphocytes from rhesus monkeys whereas 3A1 is not. Therefore, the biological effects of WT 1 could be studied in a monkey model. In a skin allograft model, WT 1 was immunosuppressive and induced a marked prolongation of graft survival.

**Keywords** monoclonal antibodies T cells leukaemia immunosuppression

## INTRODUCTION

Monoclonal antibodies (MoAbs) have now been produced against several T cell differentiation antigens. Among these are antibodies reactive with essentially all mature T cells but with only a fraction of the thymocytes (e.g. OKT1 and OKT3, reviewed by Reinherz & Schlossman, 1980). Antibody OKT11, reactive with the receptor for sheep erythrocytes (Verbi *et al.*, 1982), binds to all T cells as well as to the great majority of thymocytes, but it does not react with E rosette negative thymocytes. This E rosette negative subpopulation of thymocytes is an interesting subset with respect to thymocyte differentiation and malignancy. Cells from 10–15% of T-ALL patients (T cell origin of the cells based on reactivity with xenogeneic anti-T antiserum) do not form E rosettes (Janossy *et al.*, 1980b) and are frequently unreactive with the other OKT reagents (Greaves, 1981). Therefore, a further pan T reagent is needed which reacts strongly against these E rosette negative T lymphoid cells. WT 1 fulfills this purpose because it reacts with all thymocytes, including large blasts of putative early stages of differentiation, and also binds to the vast majority of peripheral T cells. A similar tissue distribution has been described for the antigen recognized by antibody 3A1 (Haynes, Eisenbarth & Fauci, 1979, Haynes, 1981). We found that WT 1 and 3A1 bind to the same antigen.

Correspondence Dr Wil J M Tax Department of Medicine, Division of Nephrology, St Radboudziekenhuis, Geert Grooteplein Z 8 6525 GA Nijmegen The Netherlands

since the binding of WT 1 is blocked by 3A1 In contrast to WT 1, however, 3A1 also binds to monocytes (Haynes, 1981) and acute myeloid leukaemia cells (Van Der Reijden *et al*, 1982) This reactivity appears to be not antigen specific but mediated through the Fc portion of the antibody (Haynes, 1981) There is another important difference between 3A1 and WT 1 WT 1 is reactive with T lymphocytes from rhesus monkeys, whereas 3A1 is not (Haynes *et al*, 1982) Therefore, we were able to test the biological effects of antibody WT 1 *in vivo* in monkeys The antibody proved to be immunosuppressive in a rhesus skin allograft model, and showed no toxic side effects at all in these animals Detailed studies were performed to ascertain that the reactivity of this antibody is restricted to T lineage cells in thymus and other peripheral lymphoid organs, and that the antibody does not react with bone marrow precursor cells

## MATERIALS AND METHODS

*Preparation of WT 1* Thymocytes ($10^7$) from an infant thymus which was removed during cardiac surgery, were injected intraperitoneally in Freund's complete adjuvant into a female BALB/c mouse Three weeks later, $10^7$ thymocytes of the same frozen sample were injected intravenously on 4 consecutive days before cell fusion (Tax *et al*, 1982) The spleen cells were fused with Sp2 0 in the presence of PEG 1500 as previously described (Hazekas de St Groth & Scheidegger, 1980) The antibody was purified from ascites by ammonium sulphate precipitation and protein A-Sepharose affinity chromatography The subclass of antibody WT 1 was determined by immunoelectrophoresis in agarose using subclass specific antibodies (Meloy, Springfield, Virginia, USA) and was confirmed by indirect immunofluorescence using subclass specific fluorescein conjugated antibodies (Nordic, Tilburg, The Netherlands) The light chain type of WT 1 was determined by immunoelectrophoresis using rabbit anti-lambda or -kappa chain (Litton Bionetics, Kensington, Maryland USA) Isoelectric focusing of purified WT 1 was performed on prefabricated gels on a pH gradient of 5 5–8 5 (LKB, Bromma, Sweden) and proteins were visualized by Coomassie blue staining

*Additional reagents* The MoAbs used were WT 32, an OKT3 like antibody (Tax *et al*, 1983c), UCHT 1 (another OKT3 like antibody, a gift from Dr P Beverley, Beverley & Callard, 1981), NA1/34 (which reacts with HTA-1 cortical thymocyte antigen, a gift from Dr A McMichael, McMichael *et al*, 1979), OKT11 (an antibody to the sheep erythrocyte receptor, Ortho Pharmaceutical Co, Raritan, New Jersey, USA, Verbi *et al*, 1982), WT 2 (which reacts with bone marrow precursor cells, Tax *et al*, 1982), and 3A1, generously provided by Dr B Haynes (Haynes *et al*, 1979, Haynes, 1981)

Conventional antisera were goat anti-human IgM, directly labelled with fluorescein isothiocyanate (FITC), chicken anti-HLA-DR and rabbit anti-terminal transferase (anti-TdT, Janossy *et al*, 1980a) The second layer antibodies made in goat and sheep against mouse, chicken and rabbit Ig were labelled with FITC (green) or tetramethyl rhodamine isothiocyanate (TRITC, red) and used in appropriate combinations as shown below

*Immunoprecipitation* MOLT-3 cells were surface iodinated, and lysed with 0 5% NP-40 Lysates were pre-cleared and then incubated with antibody Formalin fixed *Staphylococcus aureus* bacteria were used for the precipitation Molecular weight of the antigen recognized by WT 1 and determined by SDS-polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970)

*Cells and cell culture* Cell lines were cultured in RPMI 1640 medium (Dutch modification, containing both HEPES and sodium bicarbonate) supplemented with 7 5–15% heatinactivated fetal calf serum, 2 mM glutamine, 1mM sodium pyruvate and gentamycin (50 μg/ml) The characteristics of the lines have been described previously (Minowada *et al*, 1978) Mononuclear cells from blood and bone marrow were isolated on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) Stimulation with phytohaemagglutinin (PHA, 50 μg ml) was performed in the same medium as above except that 20% human AB serum was substituted for calf serum Granulocytes and thrombocytes were purified as described (Tax *et al*, 1982), and monocytes were obtained by the elutriation technique (De Mulder *et al*, 1981)

*Immunofluorescence and immunoperoxidase* Cells were incubated with MoAb (culture supernatant used at 1/10, or ascites at $10^{-3}$ or $10^{-4}$ final dilution), or with anti-sera (diluted 1/20- 1/40) in the presence of azide (0 1%), washed and stained with the relevant fluorochrome conjugated second layers in suspension Results of double fluorochrome staining were evaluated on a Zeiss microscope equipped with an epifluorescence attachment and selective filters for FITC and TRITC Quantitative analysis of antibody binding was performed on an EPICS V cell sorter/analyser (Coulter, Hialeah, Florida, USA) using an argon laser (power 400 mW at 488 nm) with fluorescence channel photomultiplier (tube voltage 700 V) Staining for nuclear TdT was performed on cytocentrifuge spreads prepared from suspensions of cells which had been pre-labelled for membrane markers (Janossy *et al*, 1980a) Immunoperoxidase staining was performed in sections of frozen biopsies obtained from thymus and tonsil tissues (Verbi *et al*, 1982) The incubation time for antibody labelling was 45 min to 1 h at room temperature for both first and goat anti-mouse Ig peroxidase second layers After peroxidase labelling, the sections were counterstained with Haemalum

*Enzyme linked immunoassay (ELISA)* Flat bottomed microtitre plates were coated with poly-L-lysine (0 1 mg/ml, 200 $\mu$l/well) for 30 min Each well received 100 $\mu$l cell suspension ($1–2 \times 10^6$ cells/ml, or $10^7$ thrombocytes/ml) After centrifugation (5 min 40*g*), cells were fixed with glutaraldehyde (0 025%, 15 min) and washed with PBS ELISA tests were performed with 50 $\mu$l samples of diluted culture supernatant or ascites fluid After incubation for 1 h and extensive washings with phosphate-buffered saline (PBS), pH 7, 100 $\mu$l of peroxidase-labelled rabbit anti-mouse IgG (diluted in PBS containing 1% bovine serum albumin) as added After further incubation (1 h), plates were washed and 100 $\mu$l of substrate solution (0 08% 5-aminosalicylic acid + 0 02% $H_2O_2$ in 50 mM phosphate buffer, pH 6 0) was added After 30 min, extinction at 450 nm was measured using Multiscan (Flow, Irvine, Scotland) plate reader

*Binding of $^{125}$I-labelled antibody* Purified WT 1 was labelled with $^{125}$I using the chloramine-T method (Hunter, 1973) Target cells were incubated with radiolabelled antibody for 30 min at 20 C in the presence of azide (0 1%) in a final volume of 60 $\mu$l, and washed extensively Radioactivity was counted in LKB gamma counter

In vitro *growth of bone marrow cells* Bone marrow cells were treated with antibody and rabbit complement and tested for CFU-GM, as described (Tax *et al*, 1982) The BFU-E test was adapted from Iscove, Sieber & Winterholter (1974) Orange-to-red colony bursts of at least three subclusters, or one single cluster of more than 300 cells were scored as BFU-E on day 14 of culture

*Skin grafting* Male rhesus monkeys were grafted with allogeneic skin as described elsewhere (Van Vreeswijk & Balner, 1980) Starting from 2 days prior to transplantation, treated animals received during 10 days daily injections of purified WT 1 at a dose of 1 5 mg/kg body weight

## RESULTS

*Characteristics of WT 1*

WT is an IgG2a antibody with lambda light chain It activates rabbit complement and binds to protein A Isoelectric focusing of purified antibody reveals a restricted pattern of bands near pH 6 5 (data not shown) The antigen precipitated by WT 1 has an apparent molecular weight of approximately 40,000 The diffuseness of the single peptide band suggests a high carbohydrate content (data not shown)

*Tissue section analysis*

Immunoperoxidase studies revealed that WT 1 labelled both cortical and medullary lymphocytes in the thymus, but failed to react with non-lymphoid cells such as cortical epithelium and dendritic cells in the medulla (Fig 1a) In tonsil (Fig 1b), the staining was restricted to the paracortical T cell areas and to 10–15% of cells in the germinal centres OKT11 showed the same reactivity, these positive cells are likely to be T cells (Verbi *et al*, 1982) The B blast cells in the germinal centres and B lymphocytes in the lymphocytic corona were apparently negative, and other non-T cells (e g follicular dendritic cells, endothelium, etc ) also failed to stain

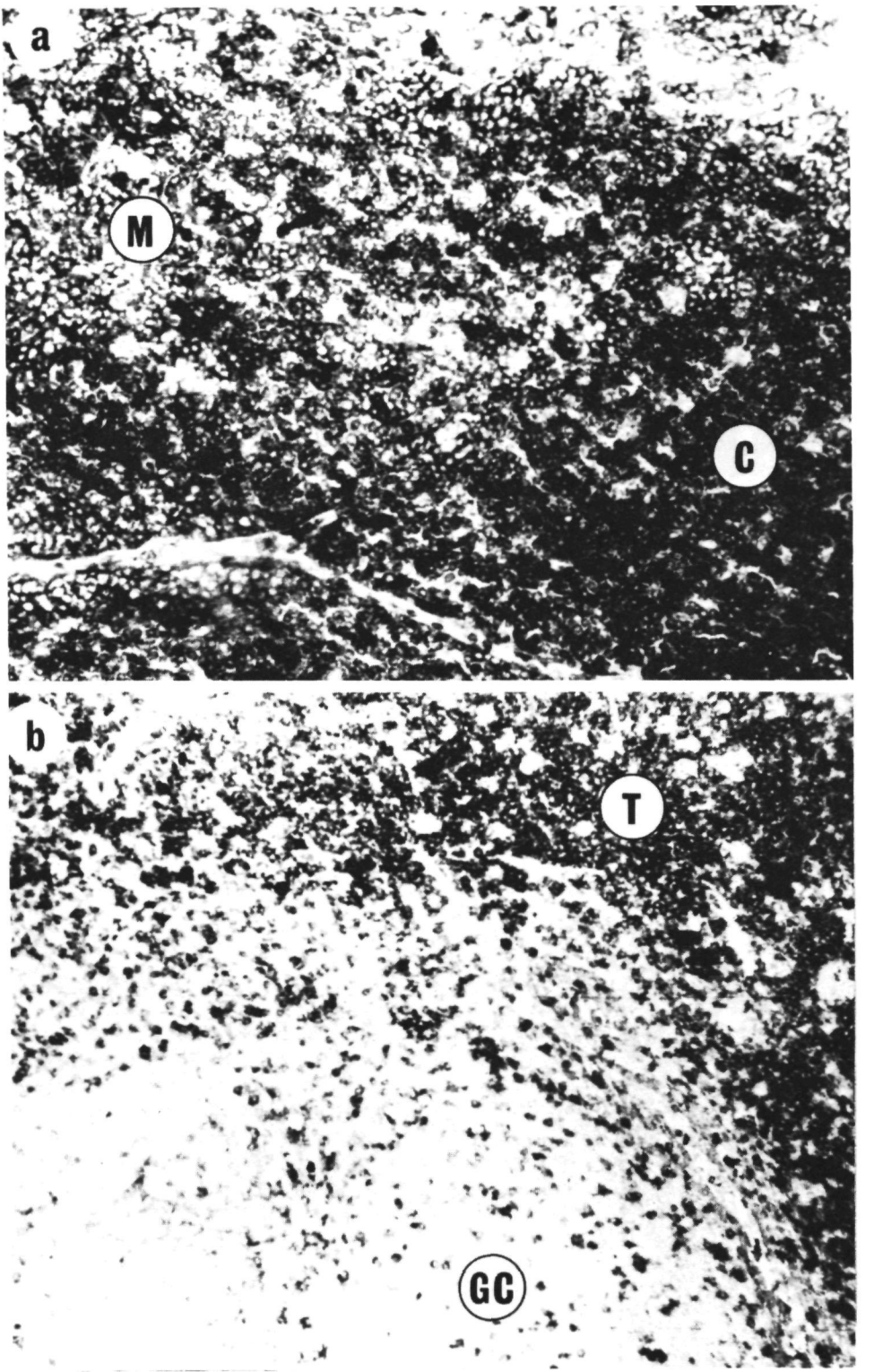
a
M
C
b
T
GC

**Table 1.** Double immunofluorescence using WT 1 and other reagents in cell suspensions

| Cell type | Combination TRITC/FITC | Percentage of cells positive*† | | | |
|---|---|---|---|---|---|
| | | +/+ | +/− | −/+ | − − |
| Blood mononuclear cells | WT 1/UCHT 1 | 76‡ | 4 | 10 | 10 |
| | WT 1/OKT11 | 79 | <1 | 3 | 18 |
| | WT 1/anti-IgM | <1 | 77 | 6 | 17 |
| | WT 1/anti-HLA-DR | <1 | 85 | 6 | 9 |
| Thymocytes | WT 1/UCHT 1 | 20 | 72 | 3 | 5 |
| | WT 1/OKT11 | 95 | <1 | <1 | 5 |
| | WT 1/anti-TdT§ | 74 | 21 | <1 | 5 |
| Tonsil cells | WT 1 UCHT 1 | 65‡ | 4 | 8 | 23 |
| | WT 1 anti IgM | 2 | 66 | 29 | 3 |
| | WT 1 anti-HLA-DR | 2 | 62 | 35 | 3 |
| Bone marrow cells | WT 1/UCHT 1 | 10 | 0 | 0 | 90 |
| | WT 1 OKT11 | 11 | <1 | <1 | 88 |
| | WT 1 anti-HLA-DR | 0 2¶ | 10 | 15 | 75 |
| | WT 1 anti-IgM | 0 6¶ | 12 | 5 | 83 |
| | WT 1/anti-TdT | 0 | 10 | 4 | 86 |

*Results of one representative experiment are shown Similar results were seen in two other tests When two MoAbs were used simultaneously, we employed subclass specific reagents (goat anti-mouse $IgG_2$ TRITC for WT 1 and anti-$IgG_1$ FITC for the other antibody) as described previously (Tidman *et al*, 1981) Antisera to IgM, HLA-DR and TdT were heterologous reagents (Janossy *et al*, 1980a)

† +/+ and −/− refer to cells which are reactive or unreactive with either antibody, +/− and −/+ refer to cells which react only with the first or second reagent, respectively

‡ Variable staining intensity

§ See Fig 2 for further detail

¶ These are minute populations which represent 2–5% of HLA-DR$^+$ cells of myeloblast morphology and 10–12% of IgM B lymphocytes in the bone marrow

*Analysis of cell suspensions*

Double label immunofluorescence studies were performed on cell suspensions from different lymphoid organs In tonsil, virtually no IgM positive B lymphocytes were WT 1 positive (only 2% double labelled cells in Table 1), confirming the tissue section analysis WT 1 positivity was confined to the UCHT 1 positive T cell population, although some of these cells stained very weakly with WT 1, There was a slight overlap between WT 1 and HLA-DR staining This apparently corresponded to the weakly HLA-DR positive, UCHT 1 positive T cells (probably activated T cells), whereas the strongly HLA-DR positive tonsil cells (B lymphocytes) were all WT 1 negative

The analysis of peripheral blood confirmed the studies on tonsil, and also showed that monocytes were WT 1 negative A few residual polymorphs present in the mononuclear cell fraction were also unreactive, a finding which was confirmed in isolated granulocyte populations from

**Fig. 1.** Analysis of sections of human thymus (a) and lymph node (tonsil, b) with WT1 using immunoperoxidase Thymocytes in both the cortical (C) and medullary (M) areas positively stain with WT 1 In the tonsil, staining is restricted to cells of the paracortical T cell area (T) and about 10% of lymphocytes in the germinal centre (GC) The staining intensity of these T cells is very variable (which is better demonstrated in sections labelled for WT 1 in immunofluorescence tests, not shown) × 200

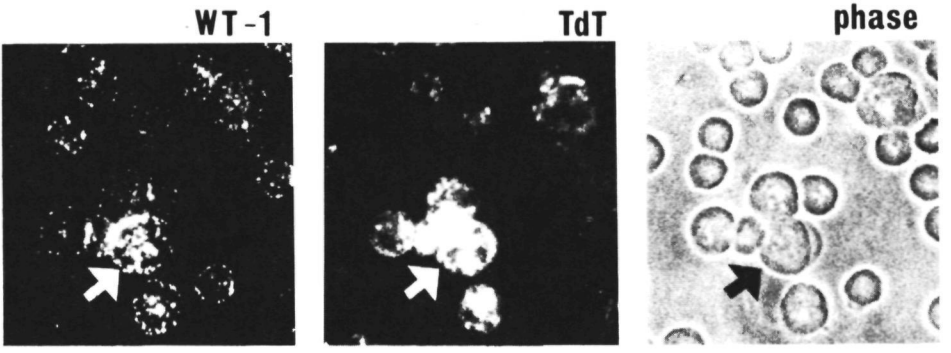


**Fig. 2.** Double labelling of thymocyte suspension with WT 1 (membrane staining with TRITC in A) and terminal transferase (TdT, nuclear staining with FITC in B). Large strongly $TdT^+$ thymic blasts are particularly intensively labelled (arrow) while small cortical thymocytes are moderately strongly positive for WT 1 ( × 650).

blood. Platelets were also WT 1 negative. The non-reactivity of WT 1 with thrombocytes and monocytes was confirmed by ELISA tests on purified cells.

In cell suspensions from infant thymus, 95% of the cells were WT1 positive, with a variable staining intensity. Flow cytometry showed that the largest T lymphocytes (1–5% of the total population) gave strong staining (data not shown). Double staining with nuclear TdT on cytospin preparations indeed confirmed that the large WT 1 positive blast cells were also strongly TdT positive (putative prothymocytes? Fig. 2). Cortical thymocytes (TdT positive cells in Fig. 2) were moderately positive with WT 1. Medullary thymocytes showed a variable staining and on some of these cells the staining was hardly detectable. Approximately 3% of thymocytes were UCHT 1 positive (peripheral type or medullary T cells) but WT 1 negative (Table 1).

Double staining revealed that TdT positive cells in the normal bone marrow were invariably WT 1 negative (Table 1). About 10% of bone marrow cells were WT 1 positive but these were mature T cells as evidenced by double labelling with UCHT 1 or OKT11 (Table 1). More than 80% of HLA-DR positive cells in bone marrow (including myeloid precursors and B lymphocytes) were WT 1 negative. Nevertheless, a small proportion of cells with myeloblast features (2–5% of large HLA-DR positive cells; 0·04–0·1% of all bone marrow cells) were WT 1 positive, albeit weakly. Similarly, 10–12% of IgM positive bone marrow cells (0·5–0·7% of all bone marrow cells) were also moderately WT 1 positive. This could have been due to the binding of goat Ig to Fc receptors. In our hands, $F(ab')_2$ reagents in general gave weaker staining than whole Ig second layers. Therefore, a very weak but genuine antigen specific reaction to a small proportion of myeloblasts and B cells could not be totally excluded.

When human cell lines were tested for their reactivity with WT 1, only T-ALL lines were found to be positive (Table 2). WT 1 was strongly positive on T blasts of all these lines (except HPB-ALL), and staining was more uniform than with OKT11. Importantly, WT 1 strongly reacted with all cells of the CCRF-CEM line, which is an E rosette negative, OKT11 negative cell type. The intensity of WT 1 staining was even more impressive when compared to the weak and variable staining seen with NA 1/34 antibody, which detects the HTA-1 (OKT6 like) cortical thymocyte antigen (Table 2). Interestingly, the common ALL cell lines (KM-3, Reh), the pre-B cell line NALM-6 and B cell lines (such as Raji and Bristol-J) as well as the only myeloid line tested (HL 60) were all negative with WT 1, confirming the T lineage specificity of this antibody.

*Antibodies WT 1 and 3A1*

The antigenic specificity of WT 1 appears to be similar to that described for antibody 3A1 (Haynes *et al.*, 1979; Haynes, 1981), although only limited information is available about the reactivity of the latter antibody with the various cell types of the bone marrow. Furthermore, the antigen precipitated by 3A1 also has a molecular weight of 40,000. Pre-incubation of T cell line Jurkat with antibody 3A1 blocked the binding of radiolabelled WT 1 as effectively as unlabelled WT 1. Similar

**Table 2.** Comparative reactivity of WT 1 and other MoAbs with human lymphoid cell lines and the HL 60 promyelocyte line

| Cell line | WT 1 | OKT11 | NA/36* (OKT6 like) |
|---|---|---|---|
| *T-ALL lines* | | | |
| Molt 3 | >99 | >99 | 65 |
| Molt-4 | >99 | 72 | 64 |
| HPB-ALL | 50 | >99 | 91 |
| HPB-MLT | >99 | 65 | 56 |
| CCRF-CEM | >99 | <1 | 32 |
| T-ALL | >99 | >99 | >99 |
| T-CLL | >99 | >99 | >99 |
| JM-1 | >99 | >99 | 63 |
| *C-ALL and pre-B lines* | | | |
| KM-3 | <1 | <1 | <1 |
| Reh | <1 | <1 | <1 |
| NALM-6 | 3 | <1 | <1 |
| *B-lines* | | | |
| Raji | <1 | <1 | <1 |
| Daudi | 12(±) | 9(±) | <1 |
| *Promyelocytic line* | | | |
| HL 60 | <1 | <1 | <1 |

* Reactive with HTA-1 cortical thymocyte antigen (McMichael *et al*, 1979)

results were obtained with another T cell line, HSB-2, and with PHA stimulated T cells (data not shown) Therefore, both WT 1 and 3A1 appear to react with the same antigen However, as will be shown below, the epitopes recognized by these antibodies are different

*Reactivity of WT 1 with rhesus lymphocytes*

In rhesus monkeys 78 ± 15% of peripheral mononuclear cells (mean ± standard deviation for six animals) were OKT11 positive, and 61% ± 10% of these T cells were reactive with WT 1 We could confirm that antibody 3A1 is not reactive with rhesus T cells (Haynes *et al*, 1982), and thus reacts with a different epitope Previous experiments in two rhesus monkeys showed that administration of WT 1 resulted in a prolonged survival of skin allografts, indicating that WT 1 has immunosuppressive activity (Jonker *et al*, 1983) We performed skin grafting in an additional rhesus monkey treated with WT 1, and found that allograft survival was also prolonged in this animal (graft survival 18 days) No toxic side-effects were observed after injection of the antibody

*Non-reactivity of bone marrow precursor cells*

In addition to the immunofluorescence studies described in *Analysis of cell suspensions*, we also performed functional assays of bone marrow cells to ascertain that the elimination of cells by WT 1 does not interfere with the outgrowth of progenitor cells Bone marrow cells were treated with WT 1 and complement (rabbit serum) and assessed for the growth of granulocyte/monocyte colony forming cells (CFU-GM) and erythroid burst forming cells (BFU-E) The growth of these precursor cells was not inhibited (Table 3) WT 2 (a MoAb directed against bone marrow precursor cells, Tax *et al*, 1982) in combination with complement abolished both CFU-GM and BFU-E completely As expected, WT 32 (OKT3 like, Tax *et al*, 1983c) plus complement had no adverse effect (Table 3)

**Table 3.** Functional analysis of bone marrow precursor cells (CFU-GM and BFU-E) after treatment with WT 1 and rabbit complement (RC)*

| Treatment | CFU-GM (%) | BFU-E (%) |
|---|---|---|
| None | 100 | 100 |
| RC | 123±19 | 130±13 |
| WT 1+RC | 124±11 | 130±39 |
| WT 32†+RC | 113±26 | 136±18 |
| WT 2‡+RC | 4±3 | 0±0 |

* Results are expressed as mean±standard deviation (two to five experiments), relative to the untreated control which was set at 100%

† WT 32 (IgG2a) shows OKT3 like reactivity (Tax *et al*, 1983c)

‡ WT 2 (IgG2a) is reactive with bone marrow precursor cells (Tax *et al*, 1982) and was used as a positive control

## DISCUSSION

WT 1 appears to be a specific reagent for T lineage cells It shows its strongest reactivity on large thymic cells and T cell lines (TdT positive cells) and on PHA activated T lymphoblasts (TdT negative cells) The antibody differs from other T specific antibodies like OKT11 (Verbi *et al*, 1982), OKT3 and OKT1 (reviewed by Reinherz & Schlossman, 1980) both in specificity and in molecular weight of the recognized antigen WT 1 is similar to antibody 3A1 (Haynes *et al*, 1979, Haynes 1981) 3A1 blocks the binding of WT 1 to T cells and therefore both antibodies appear to react with the same antigen

As contrasted with 3A1, antibody WT is reactive with the majority of rhesus T lymphocytes Therefore, we could study the biological effect of WT 1 in a rhesus model The antibody induced a prolonged survival of skin allografts in three experiments Recently, the *in vitro* effects of another 3A1 like antibody, designated 4A, were described (Morishima *et al*, 1983) When human lymphocytes were treated with antibody 4A and complement, the allogeneic *in vitro* response (MLC) was not decreased, and there was only a moderate reduction in the response to mitogens (PHA, Con A and PWM) Nevertheless, the treatment with antibody 4A plus complement eliminated both helper and cytotoxic T cell subpopulations (Morishima *et al*, 1983) After injection of WT 1 antibody, several effector mechanisms apart from complement activation may be involved in the elimination of WT 1 positive cells, e g phagocytosis and antibody-dependent cellular cytotoxicity An important aspect is that cells with low expression of 'the antigen will probably escape elimination, and after proliferation of these cells the balance between different T lymphocyte subpopulations (e g inducer and suppressor cells) may be altered Furthermore, it is conceivable that T lymphocytes are not eliminated but functionally inactivated by modulation of the WT 1 antigen Further studies will be necessary to elucidate the immunosuppressive mechanism of antibody WT 1 In view of the immunosuppressive activity of WT 1, it is of interest that this antibody binds much stronger to activated T cells than to resting T cells (Tax *et al*, 1982, Tax *et al*, 1983b)

WT 1 appears to be useful also for the diagnosis of acute leukaemia of T phenotype WT 1 invariably binds to E rosette positive T-ALL blasts, and also reacts strongly in cases of T-ALL which are E rosette negative, HDA-DR negative (Tax *et al*, 1983a) Vodinelich *et al*, 1983) The

antibody may be useful for the treatment of T-ALL (e g by removal of residual tumor cells from bone marrow followed by autologous bone marrow transplantation) Whenever a therapeutic application of a MoAb is considered, it is important to ascertain that the antibody is not reactive with bone marrow precursor cells The data presented show that WT 1 fulfills this condition

In conclusion, WT 1 is a T specific antibody which is a useful reagent for the diagnosis of T-ALL and may have therapeutic application for this type of leukemia Furthermore, the antibody has immunosuppressive activity, and may have clinical use as an immunosuppressive agent WT 1 may also yield new information of T cell function since the immunosuppressive effect suggests that the WT 1 antigen is important for T cell function

This work was supported by the Dutch Kidney Foundation and by the Leukaemia Research Fund of Great Britain We are grateful to Drs P Beverley, B Haynes, and A McMichael for providing MoAbs, and to Mrs Kamal Ivory for excellent assistance We also thank Dr M Jonker (TNO Rijswijk, The Netherlands) for helping us with the skin graft experiments We are indebted to Drs H Blacklock, H G Prentice, and other members of the Haematology Department, Royal Free Hospital, for discussions and supply of bone marrow samples, and to the surgeons in Nijmegen and at the Institute of Child Health, London, for thymus samples taken during cardiac surgery

## REFERENCES

BEVERLEY, P C L & CALLARD, R E (1981) Distinctive functional characteristics of human 'T' lympho cytes defined by E rosetting or a monoclonal anti T cell antibody *Eur J Immunol* **11,** 329

DE MULDER, P H M , WESSELS J M C , ROSENBRAND, D A , SMEULDERS, J B J M , WAGENER, D J T & HAANEN, C (1981) Monocyte purification with counterflow centrifugation monitored by continuous flow cytometry *J Immunol Meth* **47,** 31

FAZEKAS DE ST GROTH, S F & SCHEIDEGGER, D (1980) Production of monoclonal antibodies strategy and tactics *J Immunol Meth* **35,** 1

GREAVES, M F (1981) Monoclonal antibodies as probes for leukaemic heterogeneity and haemopoietic differentiation In *Leukaemia Markers* (ed by W Knapp) p 19 Academic Press, New York

HAYNES, B F (1981) Human T lymphocyte antigens as defined by monoclonal antibodies *Immunol Rev* **57,** 127

HAYNES, B F , DOWELL, B L , HENSLEY, L L GORE, I & METZGAR, R S (1982) Human T cell antigen expression by primate T cells *Science*, **215,** 298

HAYNES, B F , EISENBARTH, G S & FAUCI, A S (1979) Human lymphocyte antigens production of a monoclonal antibody that defines functional thymus-derived lymphocyte subsets *Proc Natl Acad Sci USA* **76,** 5829

HUNTER, W H (1973) Radioimmunoassay In *Handbook of Experimental Immunology* 2nd Edn (ed by D M Weir) Chap 17 Blackwell Scientific Publications, Oxford

ISCOVE, N N , SIEBER, F & WINTERHOLTER, K H (1974) Erythroid colony formation in cultures of mouse and human bone marrow analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A *J Cell Physiol* **83,** 309

JANOSSY G , BOLLUM, F J , BRADSTOCK, K F & ASHLEY, J (1980a) Cellular phenotypes of normal and leukaemic haemopoietic cells determined by selected antibody combinations *Blood*, **56,** 430

JANOSSY, G , HOFFBRAND, A V , GREAVES, M F , GANESHAGURU, K , PAIN, C , BRADSTOCK, K , PRENTICE, H G & KAY, H F M (1980b) Terminmal transferase enzyme assay and immunological membrane markers in the diagnosis of leukaemia—a multi-parameter analysis of 300 cases *Br J Haematol* **44,** 221

JONKER, M , MALISSEN, B , VAN VREESWIJK, M , MAWAS, C , GOLDSTEIN, G , TAX, W & BALNER, H (1983) *In vivo* application of monoclonal antibodies specific for human T cell subsets permits the modification of immune responsiveness in Rhesus monkeys *Transplant Proc* **15,** 635

LAEMMLI, U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T 4 *Nature* **277,** 680

MCMICHAEL, A J , PILCH, J R GALFRE, G , MASON, D Y , FABRE, J W & MILSTEIN, C (1979) A human thymocyte antigen defined by a hybrid myeloma monoclonal antibody *Eur J Immunol* **9,** 205

MINOWADA, J , JANOSSY, G , GREAVES, M F , TSUBOTA, T , SRIVASTAVA, B I S , MORIKAWA, S & TATSUMI, E (1978) Expression of an antigen associated with acute lymphoblastic leukemia in human leukemia-lymphoma cell lines *J Natl Cancer Inst* **60,** 1269

MORISHIMA, Y , KOBAYASHI, M , YANG, S Y , COLLINS, N H , HOFFMANN, M K & DUPONT, B (1982) Functionally different T lymphocyte subpopulations determined by their sensitivity to complement-dependent cell lysis with the monoclonal antibody 4A *J Immunol* **129,** 1091

REINHERZ, E L & SCHLOSSMAN, S F (1980) The differentiation and function of human T lymphocytes *Cell*, **19,** 821

TAX, W J M GREAVES, M F WILLEMS, H W LEEUWENBERG, H F M , CAPEL, P J A & KOENE, R A P

(1983a) WT 1 a monoclonal antibody reactive with T-ALL but not with other leukemias In *Modern Trends in Human Leukaemia V* (ed by R Neth, R C Gallo, M F Greaves, M A S Moore & K Winkler) p 139, Springer-Verlag, Berlin

TAX, W, JANOSSY, G, GREAVES, M, JONKER, M, WILLEMS, R, LEEUWENBERG, J, CAPEL, P & KOENE, R (1983b) WT 1 a monoclonal antibody specific for T-lineage cells In *Human leucocyte markers detected by monoclonal antibodies* (ed by A Bernard, L Boumsell, J Dausset, C Milstein & S F Schlossman) Springer-Verlag, Berlin (in press)

TAX, W J M, LEEUWENBERG, H F M, WILLEMS, H W, CAPEL, P J A & KOENE, R A P (1983c) Monoclonal antibodies reactive with OKT 3 antigen or OKT 8 antigen In *Human leucocyte markers detected by monoclonal antibodies* (ed by A Bernard, L Boumsell, J Dausset, C Milstein & S F Schlossman) Springer-Berlag, Berlin (in press)

TAX, W J M, WILLEMS, H W KIBBELAAR, M D A, DE GROOT, J, CAPEL, P J A, DE WAAL, R M W, REEKERS, P & KOENE, R A P (1982) Monoclonal antibodies human thymocytes and T lymphocytes In *Prot Biol Fluids 29th Colloquium* (ed by H Peeters) p 701 Pergamon Press, Oxford

TIDMAN, N, JANOSSY, G, BODGER, M, GRANGER, S, KUNG, P C & GOLDSTEIN, G (1981) Delineation of human thymocyte differentiation pathways utilizing double staining techniques with monoclonal antibodies *Clin exp Immunol* **45,** 457

VAN DER REIGDEN, H J, VAN RHENEN, D J, LANSDORP, P M, VAN'T VEER, M B, LANGEHUIJSEN, M M A C, ENGELFRIET, C P & VAN DER BORNE, A E G K (1983) A comparison of surface marker analysis and FAB classification in acute myeloid leukemia *Blood*, **61,** 443

VERBI, W, GREAVES, M F, KOUBEK, K, JANOSSY, G, STEIN, H, KUNG, P C & GOLDSTEIN, G (1982) OKT 11 and OKT 11A Monoclonal antibodies with pan T reactivity which block sheep erythrocyte 'receptors' on T cells *Eur J Immunol* **12,** 81

VODINELICH, L, TAX, W, BAI, Y, PEGRAM, S, CAPEL, P & GREAVES, M F (1983) A monoclonal antibody (WT 1) for detecting leukaemias of T cell precursors *Blood*, (in press)

VAN VREESWIJK, W & BALNER, H (1980) Major histocompatibility complex matching and other factors influencing skin allograft survival in related and unrelated Rhesus monkeys *Transplantation*, **30,** 196

# CHAPTER III

Induction of nonspecific cytotoxicity by monoclonal anti-T3 antibodies.

J. Immunol. 1985. 134:3770

Leeuwenberg, J., H. Spits, W. Tax, P. Capel.

0022 1767/85/1346 3770$02 00/0
THE JOURNAL OF IMMUNOLOGY

Vol 134 No 6 June 1985
Printed in U S A

# INDUCTION OF NONSPECIFIC CYTOTOXICITY BY MONOCLONAL ANTI-T3 ANTIBODIES

JET F M LEEUWENBERG,[1]* HERGEN SPITS,† WIL J. M. TAX,* AND PETER J. A CAPEL*

*From the *Department of Nephrology St Radboud Hospital University of Nymegen, 6500 HB Nymegen The Netherlands, and the †Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands*

**The effects of monoclonal anti-T3 antibodies on the effector phase of cytotoxic T lymphocytes (CTL) were studied with respect to antigen-specific and antigen-nonspecific lysis of different target cells. Anti-T3 antibodies inhibited the antigen-specific lysis by CTL generated in mixed lymphocyte cultures (MLC), but they concomitantly augmented the nonspecific killing of third-party cells such as the cell lines Daudi, Raji, and K562.**

**This nonspecific cytotoxicity was induced by various anti-T3 antibodies, whereas antibodies reactive with other antigens expressed on the cytotoxic effector cells lacked any such activity. Anti-T3 antibodies induced nonspecific cytotoxicity only when activated T cells, obtained by primary MLC, by repeated restimulation, or after cloning, were used. The antibodies had no effect on unstimulated peripheral T lymphocytes or thymocytes. The inhibition of the antigen-specific lysis and the induction of nonspecific lysis by anti-T3 was dose dependent, and both effects occurred at the same concentration range of anti-T3. $F(ab')_2$ fragments of anti-T3 inhibited the specific lysis but were not able to induce cytotoxic activity, indicating that this induction is an Fc-dependent process. When different target cells were tested, only Fc receptor-positive cells were susceptible for this nonspecific cytotoxicity. Thus, anti-T3 antibodies have a dual effect on effector CTL: they inhibit antigen-specific lysis and concomitantly induce nonspecific lysis in an Fc-dependent way.**

In an immune response, cytotoxic T lymphocytes (CTL)[2] are generated that can lyse antigen-specific target cells in the context of the major histocompatibility complex (MHC) The process of T cell-mediated cytotoxicity consists of a sequence of events starting with the recognition of target cells by CTL, leading to CTL-target cell adhesion, followed by programming of the target cell for lysis (lethal hit) After these steps the CTL-target cell conjugates dissociate, and the lytic process proceeds independently of the CTL. At the stage of conjugate formation and programming for lysis, multiple membrane elements are involved, and monoclonal antibodies (MAb) directed against these structures can interfere with the lytic process (1).

Cytolysis can be inhibited not only through blockade of antigen-specific receptor structures by anticlonotypic antibodies reactive with a T3-associated heterodimer, which is most likely the antigen receptor structure (2, 3), but also through blockade of antigens involved in conjugate formation This latter type of blockade is possible with anti-T8 MAb for $T8^+$ CTL, with anti-T4 MAb for $T4^+$ CTL (4–7), and with anti-LFA1 (8)

Anti-T3 antibodies have been found to inhibit cytolysis (9, 10) *beyond the stage of conjugate formation* OKT3 interfered with a post-recognition and adhesion step, but before the completion of the lethal hit (11, 12) This suggests that one of the components of the T3 complex, which consists of at least five subunits (13), is involved in signal processing leading to destruction of the target cell.

In addition to the inhibition of antigen-specific lysis, MAb anti-T3 induce mitosis of T lymphocytes, inhibit proliferative responses by T cells to soluble antigen and comodulate with clonotypic structures (2) These effects show that the T3 complex plays an important role in T cell function, and it is likely that T3 is a receptor-associated complex of polypeptides We have prepared anti-T3 MAb WT32 (14), similar to OKT3, Leu-4, and UCHT1 (15), as well as WT31 which recognizes a 44-kilodalton (44kd) structure from the T3 complex (16) WT31 also induces mitosis, inhibits CTL function, and inhibits binding of other anti-T3 MAb (14) We have studied the effects of anti-T3 antibodies on antigen-specific and nonspecific cytolysis Here we report that anti-T3 MAb not only inhibit the antigen-specific lysis but also induce nonspecific cytotoxicity resulting in the killing of Fc receptor-positive third-party cells

## MATERIALS AND METHODS

*Preparation of lymphocytes* Peripheral blood mononuclear leukocytes of normal healthy donors were isolated by Ficoll Paque (Pharmacia) density gradient centrifugation (17) Thymocytes were obtained from patients undergoing corrective cardiac surgery

*Monoclonal antibodies* Anti-T3 MAb used were WT31 WT32 (14) OKT3 (Ortho Diagnostics) anti Leu 4 (Becton Dickinson) UCHT1 (Beverley London) (15) SPV T3a and SPV T3b (18) The anti T8 MAb were WT81 WT82 and WT85 (14) The MAb anti-$LFA_1$ we used was SPVL1 (18) WT1 is directed against the 40kd glycoprotein T cell specific antigen (19) and 7 24 against an HLA class I common determinant

*Generation and assay of CTL* Peripheral blood lymphocytes ($10^6$)

Received for publication November 2 1984
Accepted for publication February 6 1985
The costs of publication of this article were defrayed in part by the payment of page charges This article must therefore be hereby marked *advertisement* in accordance with 18 U S C Section 1734 solely to indicate this fact
This work was supported by the Dutch Kidney Foundation
[1] Address correspondence to J Leeuwenberg Department of Nephrology St Radboud Hospital University of Nijmegen 6500 HB Nijmegen The Netherlands
[2] Abbreviations used in this paper CTL cytotoxic T lymphocyte MAb monoclonal antibody kd kilodalton PBL peripheral blood lymphocyte

were mixed with 2000 rad irradiated stimulator cells $10^5$ cells/ml RPMI 1640 (Dutch modification) supplemented with 2 mM glutamine 1 mM sodium pyruvate 50 μg/ml gentamicin and 20% heat inactivated pooled human AB serum After 6 days in culture at 37°C in the presence of 5% $CO_2$ the cells were washed and either tested for cytotoxicity or twice restimulated after 6 days with the original stimulator cells Cytotoxicity was assessed by the standard $^{51}$Cr release assay Different concentrations of effector cells were incubated in triplicate in the presence or absence of monoclonal antibodies (total volume 100 μl) in conical bottom wells (Titertek Flow Laboratories) for 30 min at 37°C before being mixed with $^{51}$Cr labeled target cells ($10^4$ cells/100 μl per well in RPMI 1640 with HFPFS supplemented with 5% human AB serum) After a 3 min 50 × G centrifugation the plates were incubated for 3½ hr at 37°C Then the plates were centrifugated at 550 × G for 10 min and 100 μl of the supernatants were removed from each well and counted in a gamma counter The percentage of specific $^{51}$Cr release was calculated by the following formula

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release in saponin} - \text{spontaneous release}} \times 100$$

The results are expressed as the percentage of specific $^{51}$Cr release or as the cytotoxicity index calculated according to the formula

$$\frac{\text{\% specific } ^{51}\text{Cr release with monoclonal antibody}}{\text{\% specific } ^{51}\text{Cr release in the absence of antibody}}$$

The results are expressed as means ± standard deviation

*CTL clone* A CTL clone (JR2 26 18 T4$^+$ T3$^+$) was obtained by limiting dilution of T cells stimulated with the Epstein Barr virus transformed B cell line JY as described (20)

*Cell lines* The cell lines used in this study were the B cell lines SB Daudi JY Raji and RPMI 1788 the T cell lines CEM and Molt 4 the null cell lines Nalm and REH and the erythromyeloid cell line K562

*F(ab')$_2$ fragments* F(ab')$_2$ fragments were prepared from WT32 antibodies after purification by protein A Sepharose CL 4B (Pharmacia) chromatography After dialysis against 0 1 M sodium acetate buffer pH = 4 50 mg purified WT32 in an end volume of 4 ml were incubated with 1 mg pepsin (Boehringer Mannheim) at 37°C for 18 hr The digest was applied to a Sephacryl 300 column (Pharmacia) to isolate the F(ab')$_2$ To eliminate contaminating traces of undigested IgG the F(ab')$_2$ was two times absorbed with protein A Sepharose The percentage of intact IgG was less than 0 03% as assessed in a radioimmunoassay

*Detection of Fc receptors* Cells ($4 \times 10^5$) were incubated with 0 5 mg/ml heat aggregated mouse IgG purified by protein A Sepharose CL 4B (Pharmacia) chromatography in an end volume of 100 μl for 30 min at room temperature After washing three times with phosphate buffered saline containing 0 2% bovine serum albumin and 0 05 $NaN_3$ the cells were incubated for 30 min at room temperature with 50 μl of 1/50 diluted fluorescein isothiocyanate labeled sheep anti mouse Ig antibodies (Cappel) This conjugate was preabsorbed with Sepharose coupled human Ig to remove unwanted cross reactivity

*Preparation of aggregated human IgG* Human IgG (Miles Laboratories Ltd ) were aggregated by heating at 63°C for 20 min at a concentration of 20 mg/ml

## RESULTS

*Effect of MAb anti T3 on the specific and nonspecific CML* CTL specific for SB cells were raised from healthy individuals Different monoclonal antibodies were tested for their ability to influence the specific lysis of SB cells and the lysis of different unrelated targets (Raji K562 Daudi) Significant inhibition of specific lysis was observed with antibodies directed against the T3 antigen (WT31 and WT32 final concentration 8 μg/ml) or against the T8 antigen (WT82 final concentration 8 μg/ml) When unrelated targets were tested however anti T3 antibodies increased the nonspecific lysis significantly An example is shown in Figure 1 The nonspecific lysis was not influenced by WT82 (Fig 1) Other antibodies directed against T8 (WT81 WT85 OKT8) against an HLA class I common determinant (7-24) or against a T cell specific antigen (WT1) also did not influence the

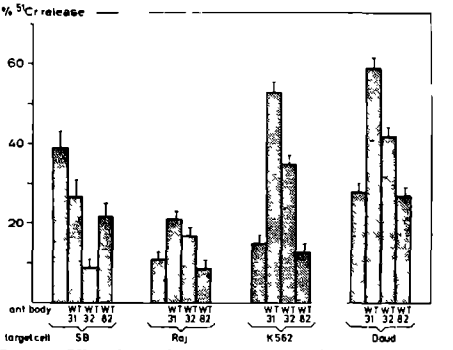


*Figure 1* Effect of anti T3 (WT31 and WT32) and anti T8 (WT82) on the specific (target = SB) and nonspecific (target = Raji K562 Daudi) cell mediated lymphoiysis MAb was added to the effector cells at a final concentration of 8 μg/ml 30 min before the addition of the $^{51}$Cr labeled target cells The target to effector ratio was 1 10 Numbers given are means ± SD of the triplicate values

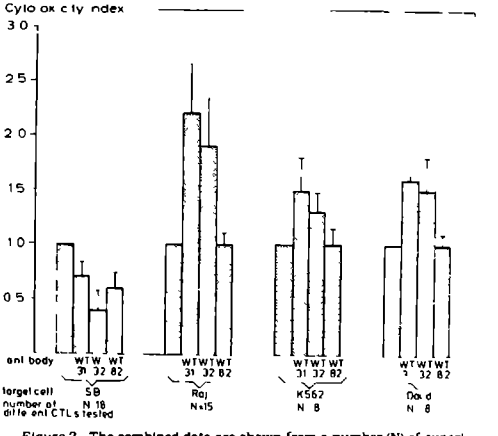


*Figure 2* The combined data are shown from a number (N) of experiments in which different CTL specific for SB cells are tested for their ability to kill different targets in the presence or absence of MAb anti T3 (WT31 and WT32 final concentration 8 μg/ml) Each bar represents the percent of lysis in the presence of MAb percent of lysis without MAb (cytotoxicity index) The target to effector ratio was 1 10

nonspecific lysis (data not shown) SB specific CTL were raised from 18 different donors by a primary mixed lymphocyte culture (MLC) of an HLA incompatible combination Part of the CTL were tested against nonspecific targets and in all cases anti T3 augmented the nonspecific lysis of the targets Raji K562 and Daudi but inhibited the lysis of SB cells (Fig 2)

*Induction of cytotoxicity by a panel of anti T3 MAb* To investigate whether all anti T3 MAb had the capacity to augment nonspecific lysis and to rule out a particular effect of a given antibody different anti T3 antibodies were investigated CTL specific for SB cells (twice stimulated) were tested against SB and Daudi cells with the following antibodies OKT3 Leu4 UCHT1 SPV-T3d and SPV T3a WT31 and WT32 WT82 and WT1 were in-

cluded as controls The results of this experiment with a target to effector ratio of 1 10 are shown in Table I The other ratios tested were 1 3 and 1 30, and at all ratios the same pattern was observed All anti-T3 and anti-T8 antibodies inhibited the specific lysis of SB but only anti-T3 enhanced the nonspecific lysis WT82 (anti-T8) and WT1 had no effect on the nonspecific lysis

*Titration of anti T3* To determine the optimal concentration of anti-T3 for inhibition of the specific lysis and for induction of nonspecific killing of Daudi cells by SB-specific CTL (twice stimulated), different concentrations of WT32 were tested ranging from 0 1 to 200 μg/ml A representative example is given at a target to effector ratio of 1 10 in Figure 3, and the results demonstrate that inhibition as well as induction of nonspecific cytotoxicity are dose dependent For most of the experiments 8 μg anti-T3/ml was chosen as the final concentration

TABLE I

*The effect of MAb on the specific and nonspecific lysis[a] by CTL anti SB (twice stimulated with irradiated SB cells*

| MAb[b] | Target Cells | | | |
|---|---|---|---|---|
| | SB | | Daudi | |
| | % Lysis | % Inhibition[c] | % Lysis | % Stimulation[d] |
| — | 33 | — | 12 | — |
| OKT3 | 20 | 39 | 30 | 150 |
| anti Leu 4 | 12 | 63 | 29 | 141 |
| WT31 | 20 | 39 | 33 | 175 |
| WT12 | 5 | 84 | 34 | 184 |
| UCHT1 | 17 | 48 | 30 | 150 |
| SPV T3d | 5 | 84 | 35 | 191 |
| SPV T3a | 4 | 87 | 24 | 100 |
| WT82 | 25 | 24 | 11 | 0 |
| WT1 | 33 | 0 | 12 | 0 |

[a] The target to effector ratio was 1 10

[b] The effector cells were incubated with the MAb 30 min before the addition of the $^{51}Cr$ labeled target cells The final concentration of monoclonal antibodies in the assay was 8 μg/ml for WT1 WT31 WT32 WT82 2 μg/ml for UCHT1 SPV T3d SPV T3a and 1 μg/ml for anti Leu 4 The commercially available OKT3 was added after dialysis at a final dilution of 1/100

[c] Percent of inhibition is expressed as $100\% - \frac{\%\ \text{lysis + Mab}}{\%\ \text{lysis − Mab}} \times 100\%$

[d] Percent of stimulation is expressed as $100\% - \frac{\%\ \text{lysis + Mab}}{\%\ \text{lysis − Mab}} \times 100\% - 100\%$

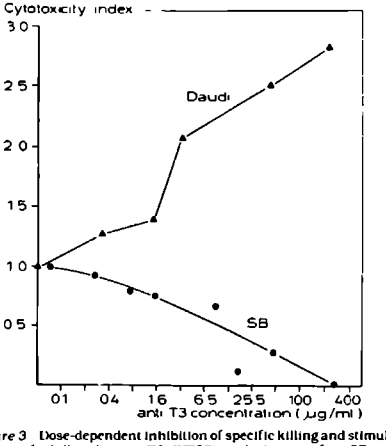


*Figure 3* Dose-dependent inhibition of specific killing and stimulation of nonspecific killing by anti T3 (WT32) with the use of an SB-specific CTL twice stimulated The target to effector ratio was 1 10

*Reactivity of unstimulated peripheral blood lymphocytes (PBL) and thymocytes* To investigate under which conditions T cells can be stimulated to nonspecific killing by anti-T3 antibodies, PBL from nine different donors were isolated These PBL were tested directly for their killing capacity of SB, Raji, Daudi, and K562 cells in the presence or absence of antibodies The monoclonal antibodies used in the assay were WT31 and WT32 (anti-T3), WT81 and WT82 (anti-T8) and WT1 in a final concentration of 8 μg/ml The combined results are shown in Figure 4 WT31 was not able to induce kill of targets by freshly isolated PBL from nine different donors, and comparable results were obtained with WT32 The other MAb (WT82, 7-24, WT1) tested also had no effect (data not shown) When these PBL were stimulated with SB cells and tested at day 6, anti-T3 inhibited the specific lysis of SB cells and induced nonspecific lysis comparable with the results given in Figure 2 (data not shown) When thymocytes were incubated with anti-T3 (anti-Leu-4 and SPV-T3d), no nonspecific lysis was induced by this treatment (data not shown)

*Fc dependency of the induced nonspecific cytotoxicity* To test whether the Fc fragments of anti-T3 antibodies are important in the induction of nonspecific lysis, $F(ab')_2$ fragments were prepared from WT32 and tested in a cell-mediated lympholysis Less than 0 03% of intact antibodies were present in this preparation as measured in a radioimmunoassay specific for Fc fragments The $F(ab')_2$ preparation had on a molar base a similar antigen-binding capacity as intact WT32 as was tested by immunofluorescence When tested with SB-specific CTL, the inhibition of the specific lysis of SB cells by $F(ab')_2$ fragments was in the same range as by WT32 on a molar base (Fig 5) The ability of $F(ab')_2$ fragments to induce nonspecific lysis was tested by using SB-specific CTL with the unrelated targets Raji Daudi, and K562 cells The results obtained with 8 μg WT32 and a similar concentration of WT32-$F(ab')_2$ (5μg/ml) are given in Figure 6, and clearly show that the induction of nonspecific lysis is Fc dependent At other target to effector ratios (1 3 and 1 30) the same pattern was observed These findings suggest the involvement of Fc receptors on the target cells in this process, and therefore a panel of different target cells was tested for the presence of Fc receptors and their susceptibility to nonspecific lysis The

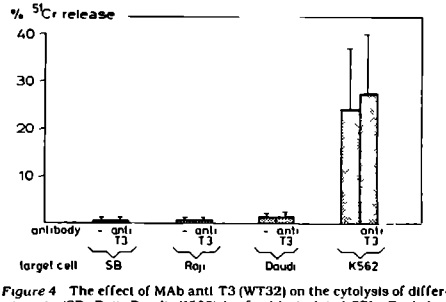


*Figure 4* The effect of MAb anti T3 (WT32) on the cytolysis of different targets (SB Raji Daudi K562) by freshly isolated PBL Each bar represents the mean percentage of lysis of nine different experiments

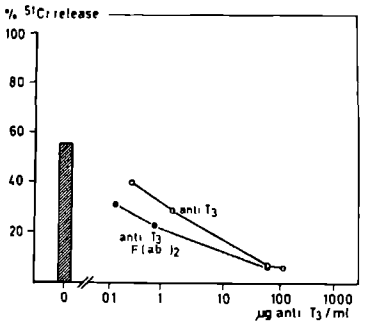


*Figure 5* Comparison of the effects of intact anti T3 (WT32) and F(ab')$_2$ fragments of WT32 on the specific lysis of SB cells by CTL specific for SB at a target to effector ratio of 1 30 The shaded bar represents the percent of lysis of SB cells without addition of antibody

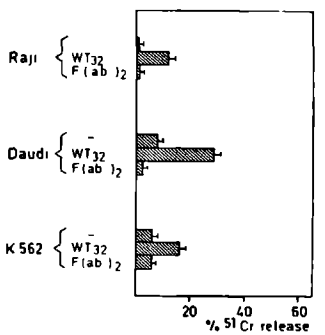


*Figure 6* Effect of anti T3 (WT32) and WT32 F(ab')$_2$ on the nonspecific lysis of Raji Daudi and K562 cells by CTL twice stimulated with irradiated SB cells at a target to effector ratio of 1 10

presence of Fc receptors was determined on different target cells by using heat-aggregated mouse IgG, and the following results were obtained the percentage of Fc receptor-positive cells was >90% for Daudi, >90% for K562, and 50% for Raji The other cells tested—RPMI 1788, CEM, Molt-4, REH, and Nalm—were mouse IgG Fc receptor negative CTL specific for SB cells were incubated with 8 μg/ml WT31 and WT32 and subsequently were tested on different target cells mentioned above Nonspecific anti-T3-induced cytotoxicity was only observed with the Fc receptor-positive cells Daudi, K562, and Raji no increase of cytotoxicity in the presence of anti-T3 was obtained with the Fc receptor-negative cells

If the Fc receptors on the target cells are involved in this process, then binding of aggregated IgG to these receptors should influence the anti-T3-induced cytotoxicity Therefore, the target cells Daudi and Raji were preincubated with aggregated human IgG before the addition to SB-specific CTL The results in Table II show that the cytotoxicity induced by WT32 (anti T3) is inhibited by the pretreatment of the target cells with aggregated human IgG

*Induction of nonspecific cytotoxicity in a T cell clone*

TABLE II

*The effect of aggregated human IgG on the anti T3 induced lysis[a] by SB specific CTL*

| Pretreatment of Target Cells[b] | Target Cells | | | |
|---|---|---|---|---|
| | % Lysis Daudi | | % Lysis Raji | |
| | — | WT32[c] | — | WT32[c] |
| — | 12 | 34 | 2 | 15 |
| Aggregated human IgG | 13 | 15 | 2 | 2 |

[a] The target to effector ratio was 1 10

[b] The target cells ($10^6$ cells/ml) were preincubated for 15 min at room temperature with heat aggregated human IgG (2 mg/ml) and diluted to the appropriate cell concentration ($10^5$ cells/ml) before addition to the CTL

[c] The effector cells were incubated with anti T3 (WT32 8 μg/ml) 30 min before the addition of the $^{51}$Cr labeled target cells

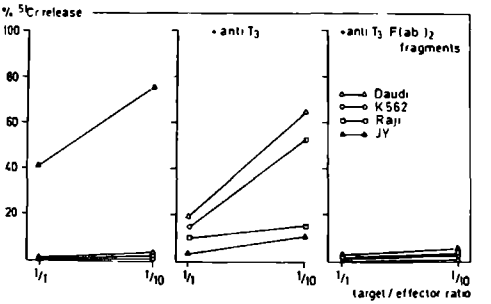


*Figure 7* Effect of anti T3 MAb (WT32) on the specific lysis of JY and the nonspecific lysis of Raji K562 and Daudi by a T cell clone (JR 2 26)

To rule out the possibility that the cytolysis induced by anti-T3 antibodies was caused by an unknown synergistic effect of different cells present in bulk T cell cultures, a cytotoxic T cell clone with a T4$^+$ T3$^+$ phenotype, reactive with JY cells, was tested At different target to effector ratios (1 1 and 1 10) killing of the targets K562, Daudi, and Raji was absent Addition of WT31 or WT32 (8 μg/ml), however, induced nonspecific lysis of these targets, whereas the killing of the stimulatory cell JY was inhibited by anti-T3 (WT31 as well as WT32) Addition of F(ab')$_2$ fragments of WT32 (5 μg/ml) did not induce lysis of K562, Daudi, or Raji cells, but it blocked the specific lysis of JY cells (Fig 7)

## DISCUSSION

The lysis of target cells by CTL is a multiple step process in which antigen is specifically recognized in the context of MHC Various differentiation antigens are involved in this process, and monoclonal antibodies against these membrane structures can inhibit the lytic process, each at different levels The inhibition of antigen-specific cytolysis by anti-T3 antibodies is at the level of programming for lysis after the adhesion step Anti-T3 antibodies have multiple effects on T cell function (13), and it is most likely that the T3 complex plays a pivotal role in signal processing The T3 complex consists of a group of proteins associated with a 44 to 49kd clonotypic heterodimer (13), which is probably the T cell receptor Our findings confirm that antigen-specific lysis by CTL is inhibited by different monoclonal antibodies, including anti-T3 antibodies (9, 10) Apart from the inhibition of the antigen specific lysis, however, anti-T3 antibodies

induced an increase in the lysis of nonspecific targets when CTL obtained from MLC were used This effect was consistently observed with CTL from 18 different donors stimulated with the B cell line SB CTL raised in MLC with different HLA mismatched leukocytes used as stimulators also mediate nonspecific cytotoxic activity in the presence of anti T3 antibodies (data not shown) This indicates that the increase in the amount of nonspecific lysis by anti T3 is a general phenomenon of CTL that is independent of the stimulator cell used to activate the CTL

Seven different anti T3 MAb with different subclasses and affinities were tested and all antibodies induced a highly significant increase in nonspecific lysis whereas three different anti 18 antibodies anti HLA A B and C and WT1 antibody were unable to enhance nonspecific cytotoxic activities These data rule out that nonspecific factors present in the antibody preparations are responsible for this effect and also show that the T3 antigen is involved in this process Various subclasses were tested as well We conclude that anti T3 independent of its subclass induces a process by which a CTL becomes cytotoxic for third party cells

Resting T cells and thymocytes were not susceptible to the induction of nonspecific lytic activity by anti T3 as was shown in Figure 4 The natural killer activity present in these preparations as defined by the lysis of K562 as target cells was not altered by anti T3 Stimulated T cells however had become susceptible to this anti T3 induced lysis when CTL were harvested at day 6 This could mean that the lytic capacity of resting T cells is not yet functional but develops in time after stimulation When cell lines were established by weekly restimulations this susceptibility for induction of cytolysis by anti T3 remained Bulk cultures of CTL or cell lines are in homogeneous cell populations and some unknown synergistic effect between different cells might be responsible for our findings To exclude this possibility a T cell clone was tested When anti T3 was added to this clone target cells that normally were not affected at all by the T cell at different effector to target ratios were lysed These data were consistent when a series of different T cell clones were used and even noncytotoxic proliferating T cell clones became cytotoxic by anti T3 incubation (21) The stimulatory concentration of anti T3 was in the same range as the inhibitory concentrations that inhibited the antigen specific killing by anti T3 The mechanism of this nonspecific lytic activity however is unknown but the results obtained with $F(ab')_2$ fragments indicate that the Fc portion is involved in this process The fact that only target cells bearing Fc receptors for mouse IgG can be lysed nonspecifically suggests that cross linking of T3 on the effector cell by Fc receptors might be involved in the triggering of a nonspecific lytic machinery in the effector cells The anti T3 mediated cytotoxicity can be inhibited by aggregated human IgG which also supports the involvement of Fc receptors in this process The anti T3 induced mitosis of T cells requires cross linking of T3 via Fc receptors (22) and immune complexes are also inhibitory in this process This parallel with our findings of the induction of nonspecific cytotoxicity by anti T3 antibodies is tempting although the concentration of anti T3 necessary for the induction of mitogenesis is much lower than the concentration to induce cytotoxicity The observed increased $Ca^{2+}$ influx induced by anti T3 in T3 positive cells (23) confirms that T3 plays a pivotal role in T cell activation and it is conceivable that T3 cross linking might indeed trigger multiple processes in T cells

In view of the induction of nonspecific lysis one would expect that inhibition of specific lysis by anti T3 MAb is counterbalanced by this process But if the presence of Fc receptors on the target cell is of importance then the antigen specific target cells used will influence the outcome of the inhibition by anti T3 Indeed the SB and JY cells which are Fc receptor negative for mouse IgG can be totally inhibited by anti T3 but the inhibition of the antigen specific lysis by anti T3 with Fc receptor positive Raji cells used is less pronounced (data not shown) In our study all target cells used were T3 negative as was confirmed by immunofluorescence also the T cell lines CEM and Molt 4 which are derived from T cells early in the differentiation pathway lacked T3 expression Therefore the anti T3 antibodies could only react with the effector cells used and induce a nonspecific cytotoxicity This effect is only observed with activated T cells and not with resting T cells Besides that already known multiple effects of anti T3 on T cells such as an increased $Ca^{2+}$ influx induction of mitosis inhibition of antigen specific proliferation and inhibition of antigen specific lysis our studies add another functional aspect of T3 in T cell activity namely the induction on nonspecific cytotoxicity The mechanism of this process is Fc dependent and most likely involves cross linking of anti T3 by Fc receptors on the target cells

*Acknowledgments* We thank Dr P Beverley for the gift of monoclonal antibody UCHT1 We thank Drs Simon Lems and Robert Koene for their support and critical reading of the manuscript

REFERENCES

1 **Martz E W Heagy and S Gromkowski** 1983 The mechanism of CTL mediated killing monoclonal antibody analysis of the roles of killer and target cell membrane proteins *Immunol Rev* 72 73
2 **Meuer S C K A Fitzgerald R E Hussey J C Hodgdon S F Schlossman and E L Reinherz** 1983 Clonotypic structures involved in antigen specific human T cell function Relationship to the T3 molecular complex *J Exp Med* 157 705
3 **Lancki D W M I Lorber M R Loken and F W Fitch** 1983 A clone specific monoclonal antibody that inhibits cytolysis of a cytolytic T cell clone *J Exp Med* 157 921
4 **Terhorst C A van Agthoven E Reinherz and S Schlossman** 1980 Biochemical analysis of human T lymphocyte differentiation antigen T4 and T5 *Science* 209 520
5 **Spits H H Yssel C Terhorst and J E De Vries** 1982 Establishment of human T lymphocyte clones highly cytotoxic for an EBV transformed B cell line in serum free medium Isolation of clones that differ in phenotype and specificity *J Immunol* 128 95
6 **Spits H J Borst C Terhorst and J E De Vries** 1982 The role of T cell differentiation markers in antigen specific and lectin dependent cellular cytotoxicity mediated by T8 and T4 human cytotoxic T cell clones directed at class I and class II MHC antigens *J Immunol* 129 1563
7 **Flomenberg N K Naito E Duffy R Knowles R Evans and B Dupont** 1983 Allocytotoxic T cell clones both Leu2 3 and Leu2 3+ T cells recognize class I histocompatibility antigens *Eur J Immunol* 13 905
8 **Sanches-Madrid F A M Krensky C F Ware E Robbins J L Strominger S J Burakoff and T A Springer** 1982 Three distinct antigens associated with human T lymphocyte mediated cytolysis LFA 1 LFA 2 and LFA 3 *Proc Natl Acad Sci USA* 79 7489
9 **Chang T W P C Kung S P Gingras and G Goldstein** 1981 Does OKT3 monoclonal antibody react with an antigen recognition structure on human T cells? *Proc Natl Acad Sci USA* 78 1805
10 **Platsoucas C D and R A Good** 1981 Inhibition of specific cell

mediated cytotoxicity by monoclonal antibodies to human T cell antigens *Proc Natl Acad Sci USA 78 4500*

11 **Landegren U U Ramstedt I Axberg M Ullberg M Jondal and H Wigzell** 1982 Selective inhibition of human T cell cytotoxicity at levels of target recognition or initiation of lysis by monoclonal OKT3 and Leu 2a antibodies *J Exp Med 155 1579*

12 **Tsoukas C D D A Carson S Fong and J H Vaughan** 1982 Molecular interactions in human T cell mediated cytotoxicity to EBV II Monoclonal antibody OKT3 inhibits a post killer target recognition/adhesion step *J Immunol 129 1421*

13 **Terhorst C** 1984 Cell surface structures involved in human T lymphocyte specific functions analysis with monoclonal antibodies In *Receptors and Recognition* M Greaves ed Chapman and Hall London P 131

14 **Tax W J Leeuwenberg H Willems P Capel and R Koene** 1984 Monoclonal antibodies reactive with OKT3 antigen or OKT8 antigen In *Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies* Springer Verlag Berlin P 721

15 **Beverley P C and R E Callard** 1981 Distinctive functional characteristics of human "T" lymphocytes defined by E rosetting or a monoclonal anti T cell antibody *Eur J Immunol 11 329*

16 **Oettgen H C J Kappler W Tax and C Terhorst** 1984 Characterization of the two heavy chains of the T3 complex on the surface of human T lymphocytes *J Biol Chem 259 12039*

17 **Boyum A** 1968 Isolation of mononuclear cells and granulocytes from human blood Isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at 1 g *Scand J Clin Lab Invest (Suppl) 21 77*

18 **Spits H G Keizer J Borst C Terhorst A Hekman and J De Vries** 1983 Characterization of monoclonal antibodies against cell surface molecules associated with cytotoxic activity of natural and activated killer cells and cloned CTL lines *Hybridoma 2 423*

19 **Tax W N Tidman G Janossy L Trejdosiewicz R Willems J Leeuwenberg T De Witte P Capel and R Koene** 1984 Monoclonal antibody (WT1) directed against a T cell surface glycoprotein characteristics and immunosuppressive activity *Clin Exp Immunol 55 427*

20 **Spits H J Borst M Giphart J Coligan C Terhorst and J De Vries** 1984 HLA DC antigens can serve as recognition elements for human cytotoxic T lymphocytes *Eur J Immunol 14 299*

21 **Spits H H Yssel J Leeuwenberg and J De Vries** 1985 Antigen specific cytotoxic T cell and antigen specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3 Eur J Immunol *15 88*

22 **Tax W F Hermes R Willems P Capel and R Koene** 1984 Fc receptors for mouse IgG1 on human monocytes polymorphism and role in antibody induced T cell proliferation *J Immunol 133 1185*

23 **O Flynn K E D Zanders J R Lamb P C L Beverley D L Wallace P E R Tatham W J M Tax and D C Linch** 1985 Investigation of early T cell activation analysis of the effect of specific antigen IL 2 and monoclonal antibodies on intracellular free calcium concentration *Eur J Immunol 15 7*

# CHAPTER IV

**Antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3.**

**Eur. J. Immunol. 1985. 124:2708**

**Spits, H., H. Yssel, J. Leeuwenberg, J. De Vries.**

Hergen Spits*,
Hans Yssel*,
Jet Leeuwenberg* and
Jan E. De Vries*

Division of Immunology, Netherlands Cancer Institute*, Amsterdam and Department of Medicine, Division of Nephrology, Nijmegen

# Antigen-specific cytotoxic T cell and antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3

T3 is a human differentiation antigen expressed exclusively on mature T cells. In this study it is shown that anti-T3 monoclonal antibodies, in addition to their capacity to induce T cells to proliferate, are able to induce antigen-specific cytotoxic T lymphocyte clones to mediate antigen nonspecific cytotoxic activity. It is furthermore shown that anti T3 reagents are able to trigger lytic activity in T cell clones characterized as noncytotoxic antigen-specific proliferating T cells. The data presented indicate that perturbation of T3 can trigger the lytic machinery in cytolytic as well as noncytolytic T cell clones.

## 1 Introduction

T cell activation requires the interaction between antigen and products of the major histocompatibility complex (MHC) on the antigen-presenting cell and the T cell receptor for antigen on the T cell. The activation of T cells by antigen can be mimicked by monoclonal antibodies (mAb) against the T cell antigen T3 [1–3]. This is understandable in view of the findings that the T cell receptor for alloantigen [4, 5] and for antigen plus a self MHC product [6] are disulfide-linked heterodimers with a molecular weight of 90 000 which are associated with nonvariable proteins of the T3 complex [7, 8]. In this study we demonstrate that in addition to the induction of T cell proliferation [1, 2] and inhibition of antigen specific cytotoxic T cell activity [9–13] anti-T3 reagents are also able to induce nonspecific cytotoxic activity, even in antigen-specific proliferative T cell clones.

## 2 Materials and methods

### 2.1 T cell clones and cell lines

The characteristics of the T cell clones used in this study are shown in Table 1. The alloreactive cytotoxic and proliferating T cell clones used were isolated from T cells of donor HG (HLA-A2, w24, B40, w44, DRw6,7), Liv (HLA-2, w31, Bw44, w60, DR2,-), and JR (HLA-Aw23, w29, B7, 7, DR5) which were stimulated with cells of the Epstein-Barr virus (EBV)-transformed B cell line JY as described previously [14]. Cytotoxic T lymphocyte (CTL) clone HG-61 ($T3^+T4^-T8^-$) lyses 30% of B7, 50% of B40 and 100% of B27 (subtype k)-bearing target cells [15]. Its reactivity is blocked by the class I MHC-specific antibody W6/32. CTL clone Liv 8 ($T3^+T4^-T8^-$) recognizes 90% of all cells bearing B7. Its specificity, as far as tested, is identical to that of CTL clone HG-31, which has been analyzed previously to be specific for HLA-B7 [16]. The activity of CTL clone Liv-8 was blocked with W6/32. The CTL clone JR-2-26 killed exclusively cells expressing HLA-DRw6 [17]. Its activity was blocked by the mAb SPV-L3, which recognizes a monomorphic determinant present on HLA-DC molecules, indicating that JR-2-26 recognizes an HLA-DC product [17]. The proliferating T lymphocyte (PTL) clone JR-2-3 proliferated only with JY cells and with most but not all cells expressing the HLA-DRw6 specificity (H. Spits, unpublished). The PTL clones HG-49 and HG-120 proliferated after stimulation with irradiated JY. This proliferation could be blocked with the anti-HLA-DR mAb Q5/13 (results not shown). The specificity of these clones was not investigated further. The clones MK-2, MK-6, MK-9 and MK 21 which were all of the $T4^+T8^-$ phenotype were derived from T cells of donor MK (HLA-A2, 29, Bw44, w22, Cw1, DR5 7) who was immunized intramuscularly with 1 mg *Helix pomata* hemocyanin (HPH, kindly provided by Drs. Torensma and The, Department of Clinical Immunology of the State University, Groningen, The Netherlands). Fourteen days after immunization $10^6$ peripheral blood lymphocytes/ml were stimulated *in vitro* with 50 µg/ml HPH. Six days later the cells were cloned by limiting dilution at one cell every 2 wells using irradiated (4000 rds) autologous and allogeneic lymphocytes ($10^6$ ml) and irradiated (5000 rds) JY cells, 10 µg/ml HPH and 5% (v/v) of an interleukin 2 (IL2)-containing supernatant. When necessary, T cell clones were subcloned using the same procedure. Cloning and subcloning of the cells was performed in flat-bottomed 96-well microtiter plates (no. 3596 Costar Cambridge, MA). Proliferating cells became visible at day 10–14.

**Table 1.** Summary of the properties of the T cell clones used in this study

| T cell clone | Phenotype | Function | Specificity associated with |
|---|---|---|---|
| HG-49 | $T4^+T8^-$ | PTL[a] | – |
| HG-61 | $T4^-T8^+$ | CTL[b] | HLA-B27k |
| HG-120 | $T4^+T8^-$ | PTL | – |
| JR-2-3[c] | $T4^+T8^-$ | PTL | HLA-DRw6 |
| JR-2-26 | $T4^+T8^-$ | CTL | HLA-DRw6 |
| Liv-8 | $T4^-T8^+$ | CTL | HLA-B7 |
| MK-2 | $T4^+T8^-$ | PTL | HPH |
| MK-6 | $T4^+T8^-$ | PTL | HPH |
| MK-9 | $T4^-T8^-$ | PTL | – |
| MK-21[c] | $T4^+T8^-$ | PTL | HPH |

a) Proliferating non-CTL clone
b) CTL clone
c) The PTL clones JR-2.3 and MK 21 were derived from subcloning the original JR-2.3 and MK 21 parent clones

**Correspondence.** Hergen Spits, Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, NL.

**Abbreviations:** **CTL:** Cytotoxic T lymphocyte. **PTL:** Proliferating T lymphocyte. **EBV:** Epstein-Barr virus. **HPH:** *Helix pomata* hemocyanin. **BSA:** Bovine serum albumin. **TT:** Tetanus toxoid. **MHC:** Major histocompatibility complex. **mAb:** Monoclonal antibody(ies).

0014-2980/85/0101-0088$02.50/0

and were transferred to 24-well plates (no. 3424, Costar) and expanded by stimulating the clones once or twice in 14 days with the feeder cell mixture which was used to clone the cells. Every 3 days the cultures were split and fresh medium containing 5% (v/v) of an IL 2-containing supernatant [14] was added. The cloned T cells were used in the cytotoxic assays *(vide infra)* 9 days after stimulation with the feeder cell mixture. Cloning procedure and tissue culture were carried out in serum-free Iscove's medium supplemented with $2 \times 10^{-5}$ M ethanolamin (Merck, Darmstadt, FRG), 35 μg/ml transferrin, 5 μg/ml insulin and 2.5 mg/ml bovine serum albumin (BSA; all three from Sigma, St. Louis, MO) and 1% (v/v) of a dispersion of lipids extracted from human plasma [14]. The EBV-transformed B cell lines JY and Laz 475 (kindly provided by Dr. H. Lazarus, Dana-Farber Cancer Center, Boston, MA) were cultured in serum-free Iscove's medium.

### 2.2 mAb

The mAb anti-Leu-4 ($IgG_1$), which recognizes the T cell antigen T3 [18] was obtained from Becton Dickinson (Mechelen, Belgium); mAb SPV-T3b ($IgG_{2a}$), SPV-L1 ($IgG_1$) and SPV-T8 ($IgG_1$) which recognize T3, LFA-1 and T8, respectively, have been described previously [19, 20]. SPV-L3 ($IgG_{2a}$) recognizes a nonpolymorphic determinant present on HLA-DC products [17]. W6/32 ($IgG_{2a}$), obtained from Sera Labs., Crawley Down, GB, recognizes a determinant present on all HLA-A, B and C molecules [21]. In some experiments the mAb were partly purified by precipitation at 45% $(NH)_2SO_4$ saturation, followed by dialysis against 2 volumes of phosphate-buffered saline.

### 2.3 Cytotoxicity assays

The assay measuring inhibition or induction of cytotoxic activity was carried out as follows: the T cell clones were preincubated for 30 min at 37 °C in U-shaped microtiter wells with mAb in 50 μl Iscove's medium supplemented with 0.25% BSA. Then 50 μl containing $10^3$ $^{51}Cr$-labeled target cells were added. The plates were centrifuged at $50 \times g$ for 5 min and incubated for 4 h at 37 °C and 5% $CO_2$. The percentage cytotoxicity was calculated as described previously [14].

### 2.4 Measurement of proliferation

The antigen specificity of cloned T cells was determined by measuring the proliferation of $5 \times 10^3$ cloned T cells/well in the presence of $2 \times 10^3$ autologous monocytes/well and various antigens or irradiated (5000 rds) JY cells ($2 \times 10^3$ cells/well). These tests were carried out in flat-bottomed 0.2 ml wells (Costar) in serum-free Iscove's medium. Three days later 0.4 μCi (= 14.8 kBq) [$^3$H]dThd was added and after overnight incubation, the cultures were harvested on glass fiber filter strips. After drying, the filters were counted in a liquid scintillation counter.

## 3 Results

### 3.1 Induction of antigen nonspecific cytotoxicity by anti-T3 mAb

The effect of two different anti-T3 antibodies, anti-Leu4 and SPV-T3b, on the cytotoxic activity of two CTL clones, Liv-8 and HG-61, was investigated. Table 2 shows that the cytotoxic activity of CTL clone HG-61 against JY cells was blocked by both anti-T3 antibodies. The cytotoxicity, however, of another CTL clone Liv-8 was not or only minimally affected, when compared with the effect of the anti-T3 antibodies on the activity of HG-61. The capacity of the anti-T3 reagents to block the activity of HG-61 and Liv-8 could not be enhanced by increasing the concentration of the antibodies (results not shown). Furthermore, it was found that these two clones HG-61 and Liv-8, which were not cytotoxic for the B cell line Laz 475, could kill this line efficiently in the presence of the anti-T3 mAb (Table 2). Although the induction of cytotoxicity by anti-T3 mAb showed some degree of variation, the strongest cytotoxic activity was induced in CTL clone Liv-8. The antigen-specific cytotoxicity of Liv-8 against JY cells was minimally blocked by the anti-T3 reagents. In contrast the activity of HG-61 was blocked well by the anti-T3 antibodies, but these reagents were less effective in inducing cytotoxicity of HG-61 against Laz 475. Anti-T3 antibodies did not only induce cytotoxicity in $T8^+$ CTL clones, but also in the $T4^+$ CTL clone JR-2-26, which is specific for a HLA-DC product present on HLA-DRw6-positive cell lines (Table 3). Moreover, Table 3 shows that the antibodies against other cell surface antigens, which are involved in cytotoxic activity, SPV-L1, SPV-T8, OKT4a, W6/32 and SPV-L3, are unable to induce

**Table 2.** The effect of two anti-T3 mAb on the cytotoxic activity mediated by the CTL clones HG-61 and Liv-8[a)]

| mAb | % Cytotoxicity Target cell | | | |
|---|---|---|---|---|
| | JY | | Laz 475 | |
| | Liv-8 | HG-61 | Liv-8 | HG-61 |
| None | 59 | 85 | 3 | 3 |
| Anti-Leu-4 | 54 | 55 | 63 | 27 |
| SPV-T3b | 42 | 37 | 50 | 14 |

a) The commercially available stock solution of anti-Leu-4 (100 μg/ml) was diluted 1 : 100 to give a final dilution of 1 μg/ml, whereas SPV-T3b was used as ascites in a final dilution of 1 : 100. The E/T ratio was 5 : 1.

**Table 3.** The effect of mAb on the cytotoxic activity of HG-61 and Liv-8 and JR-2-26 against the B cell line Laz 475[a)]

| mAb | Specificity | % Cytotoxicity | | |
|---|---|---|---|---|
| | | HG-61 ($T4^-T8^+$) | Liv-8 ($T4^-T8^+$) | JR-2-26 ($T4^+T8^-$) |
| None | – | 4 | 2 | 6 |
| Anti-Leu-4 | T3 | 40 | 54 | 30 |
| OKT-4A | T4 | 0 | 4 | 13 |
| SPV-T8 | T8 | 6 | 0 | 3 |
| SPV-L1 | LFA-1 | 0 | 4 | 2 |
| W6/32 | HLA-A, B, C | 2 | 2 | 4 |
| SPV-L3 | HLA-DC | 9 | 4 | 6 |

a) The concentrations of added antibodies were 2 μg/ml, except SPV-T8 which was added as a 1/100 dilution of ascites. The E/T ratio used was 5 : 1.

cytotoxicity in these CTL clones, indicating that the cytotoxicity inducing capacity of anti-T3 antibodies is a specific effect.

### 3.2 Induction of cytotoxic activity in noncytotoxic T cell clones

Since the ability to lyse target cells is thought to be absent in noncytotoxic antigen-specific proliferating T cells, anti-T3 antibodies should not be able to render such T cell clones cytotoxic. However, to our surprise anti-Leu-4 was able to trigger significant cytotoxicity in clones characterized as antigen-specific proliferating T cells. In Table 4 it is shown that 2 μg/ml anti-Leu-4 induced cytotoxicity in four noncytotoxic $T4^+$ clones which were derived from T cells of a donor immunized with the antigen HPH. Three of these clones proliferated specifically in response to HPH in the presence of autologous monocytes, but not in response to the control antigen tetanus toxoid (TT; Table 4). Note that one of these clones was subcloned (MK-21). Anti-Leu-4 also triggered cytotoxicity in the T cell (sub)clone JR-2-3, which proliferates in response to irradiated JY cells in the absence of exogenous IL 2, but which was not cytotoxic for JY or for other target cells, including K562 cells which are highly sensitive for natural killer-like activity (results not shown). However, anti-Leu-4 did not trigger cytotoxic activity in all $T3^+$ cells, since two $T3^+$ PTL clones, HG-49 and HG-120, were not cytotoxic in the presence of anti-Leu-4 (Table 4).

**Table 4.** Anti-Leu-4 induces noncytotoxic antigen-specific T cell clones to express cytotoxic activity against Laz 475 cells

| T cell clone | [$^3$H]dThd[a] incorporation (cpm × $10^{-3}$) after stimulation with | | | | Function | % Cytotoxicity[b] | |
|---|---|---|---|---|---|---|---|
| | None | HPH[c] (1 μg/ml) | TT[d] (15 LF/ml) | JY | | Medium | Anti-Leu-4 |
| HG-61 | – | NT[e] | NT | NT | Cytotoxic | 2 | 27 |
| JR-2-3 | 1 | NT | NT | 6 | Noncytotoxic | 3 | 36 |
| MK-2 | 0.9 | 11 | 0.5 | NT | Noncytotoxic | 3 | 32 |
| MK-6 | 0.5 | 10 | 0.6 | NT | Noncytotoxic | 2 | 28 |
| MK-9 | 0.9 | 0.5 | 0.5 | NT | Noncytotoxic | 2 | 40 |
| MK-21 | 0.6 | 18 | 0.6 | NT | Noncytotoxic | 4 | 38 |
| HG-49 | 0.5 | NT | NT | 4 | Noncytotoxic | 5 | 8 |
| HG-120 | 0.8 | NT | NT | 8 | Noncytotoxic | 0 | 4 |

a) [$^3$H]dThd = Tritiated thymidine.
b) Determined at an E : T ratio of 10 : 1.
c) 1 μg/ml.
d) 15 LF/ml.
e) NT = Not tested.

**Table 5.** The effect of antibodies against LFA-1 and T8 on the cytotoxic activity of clone HG-61 and Liv-8 against Laz 475 in the presence of anti-Leu-4[a]

| CTL clone | % Cytotoxicity in the presence of 2 μg/ml anti-Leu-4 | | | | |
|---|---|---|---|---|---|
| | Control | mAb added | Reciprocal dilution of ascites | | |
| | | | 50 | 200 | 800 |
| HG-61 | 25 | SPV-L1 | 5 | 7 | 10 |
| | | SPV-T8 | 33 | 32 | 30 |
| Liv-8 | 40 | SPV-L1 | 4 | 13 | 17 |
| | | SPV-T8 | 50 | 53 | 43 |

a) The cytotoxic activity of clone HG-61 and Liv-8 against Laz 475 in the absence of anti-Leu-4 was 2 and 1%, respectively. The E/T ratio used was 5 : 1.

### 3.3 The effect of mAb against LFA-1 and T8 on the anti-T3-induced cytotoxicity

The cytotoxicity induced by anti-Leu-4 was strongly blocked by SPV-L1, an antibody which recognizes human LFA-1 [19, 20] (Table 5), but not by SPV-T8 which is an antibody against T8 [19]. Since LFA-1 is thought to be involved in adhesion between the effector and the target cell [22], this finding suggests that cell-cell contact is required for the anti-T3-induced cytotoxicity to occur. This notion is supported by the finding that EDTA which blocks conjugate formation [23] also inhibits the killing activity induced by anti-Leu-4 (results not shown). Furthermore, these results exclude the possibility that the target cells are lysed by a soluble lymphotoxin in the absence of effector-target cell binding.

## 4 Discussion

Two novel points emerge from the study presented in this study. First, perturbation of T3 by anti-T3 mAb results in the induction of antigen nonspecific cytotoxic activity, and second, anti-T3 reagents may trigger a cytolytic mechanism in T cell clones characterized as noncytotoxic antigen-specific proliferating T cells.

Although only a small number of clones was analyzed here, the observations suggest that anti-T3 induces cytotoxicity in T cell clones irrespective of whether the T cell clone was cytotoxic or not, since noncytotoxic antigen-specific proliferating T cell clones became cytotoxic in the presence of anti-Leu-4. Nevertheless not all T cell clones were induced by anti-T3 antibodies, since two proliferating T cell clones could not be rendered cytotoxic by anti-T3. Since the mechanism of the anti-T3-induced cytotoxicity is not understood, we do not know why some clones are rendered cytotoxic by anti-T3 antibodies and others not. It is possible that the clones, which can be triggered to express cytolytic activity, represent a subset of T cells which is different from those which cannot be triggered by anti-T3 reagents. On the other hand, it may be that PTL clones can only be induced to mediate cytolytic activity at a certain stage of maturation. It has been reported by several groups that antigen-specific CTL clones acquire nonspecific cytotoxic activity after a prolonged period of culture [24–26]. This phenomenon has been termed aged killer activity by Simons et al. [26], and indicates that "aging" of T cell cultures may result in changing properties of the T cells [27]. Furthermore, it has been reported that T cell clones mediating allospecific cytotoxicity may also have proliferative capacity as well as helper activity for B cell differentiation [28, 29]. Thus there appears to be an overlap between antigen-specific cytotoxic and proliferating capacity. Such a conclusion can also be drawn from the findings of Fleischer who reported that proliferating clones specific for virus in the context of self class II MHC products acquire cytotoxic activity with the same specificity after prolonged culture of the T cell clones [30].

However, the cytotoxicity induced by anti-T3 antibodies is not dependent on the age of the T cell clones, since the clones HG-49 and HG-120, which could not be induced to become cytotoxic, were of the same age as the inducible clones HG-61 and MK-9. In addition, cytotoxicity could be induced both in relatively young clones (less than 4 weeks in culture) as well as in relatively old clones (11 weeks in culture).

Since it has been found that anti-LFA-1 antibodies block cell-cell adhesion in the murine [22] as well as in the human system (H. Spits, unpublished), the observation that SPV-L1 inhibits the anti-T3 induced cytotoxicity indicates that cell-cell contact is required. It is not yet understood how contact between the T cell clone and the target cell in the absence of specific antigen recognition is established. Recently it has been found that $F(ab')_2$ fragments of anti-T3 antibodies, which are thoroughly depleted of intact antibodies, fail to induce nonspecific cytotoxicity, whereas they effectively block antigen-specific cytotoxic activity (P. Capel, personal communication). This suggests that binding of the T cell clone to the target cell involves binding of the target cell to the Fc part of the anti-T3 antibody bound to the effector cell.

The mechanism by which anti-T3 antibodies induce cytotoxicity is presently unclear. It may be possible that T3 is directly involved in the triggering of the lytic machinery. This interpretation is supported by the observations that anti-T3 reagents block antigen-specific cytotoxicity not by preventing the formation of stable conjugates, but in a later stage of the cytotoxic reaction [12, 13]. The fact that the receptor for alloantigen is associated with the T3 complex [4, 8] raises the possibility that one of the components of the T3 complex converts the signal provided by the binding of antigen to its receptor to trigger cytotoxicity. It seems that binding of anti-T3 antibodies to their determinants on one hand prevents the transduction of the cytotoxicity inducing signal provided by the binding of antigen to its receptor, but that these antibodies on the other hand can deliver a signal which triggers cytolysis. The cytotoxic activity induced by anti-T3 is generally much less than the one induced by alloantigen and, therefore, the resultant effect of anti-T3 on the cytotoxic activity of T cell clones is partial inhibition.

The induction of cytotoxicity by anti-T3 reagents resembles lectin-dependent cellular cytotoxicity (LDCC). Phytohemagglutinin (PHA) and concanavalin A (Con A) induce antigen-specific CTL clones to mediate nonantigen-specific cytotoxicity [10]. This effect of PHA and Con A is not merely a consequence of bridging together target cells and effector cells, because wheat germ agglutinin (WGA) which agglutinates target cells and effector cells is not able to induce LDCC [31]. Since PHA and Con A inhibit the binding of anti-T3 reagents [32] and WGA does not (H. Spits, unpublished), it is likely that PHA and Con A bind to components of T3. Therefore, it may well be possible that (part of) LDCC activity is caused by triggering of T3 by Con A and PHA. The finding reported by Wee and Bach [33] that some but not all PTL clones mediate LDCC stresses the similarity between anti-T3-dependent cytotoxicity and LDCC, since anti-T3 does not induce cytotoxicity in all PTL clones (Table 4).

The demonstration that T3 plays a role in the triggering of cytotoxic activity opens a way to study the molecular aspects of the mechanism of the "programming for lysis" and "lethal hit" stages of the cytotoxic reaction.

*We thank A. Voordouw for technical assistance, Drs. P. Capel, W. Tax and Jannie Borst for their critical reading of the manuscript and Marie Anne van Halem for secretarial help.*

Received May 29, 1984, in revised form August 23, 1984.

## 5 References

1 Van Wauwe, J. P., De Mey, J. R. and Goossens, J. G., *J. Immunol.* 1980 *124* 2708.
2 Burns, G. F., Boyd, A. W. and Beverley, P. C. L., *J. Immunol.* 1982 *129* 1451.
3 Reinherz, F. L., Hussey, R. E. and Schlossman, S. F., *Eur. J. Immunol.* 1980 *10* 758.
4 Meuer, S. C. and Fitzgerald, E. L., *J. Exp. Med.* 1983 *157* 705.
5 Meuer, S. C., Acuto, O., Hussey, R. E., Hogdon, J. C., Fitzgerald, K. A., Schlossman, S. F. and Reinherz, E. L., *Nature* 1983 *303* 808.
6 Meuer, S. C., Cooper, D. A., Hogdon, J. C., Hussey, R. E., Fitzgerald, K. A., Schlossman, S. F. and Reinherz, E. L., *Science* 1983 *222* 1239.
7 Borst, J., Prendiville, M. A. and Terhorst, C., *J. Immunol.* 1982 *128* 1560.
8 Reinherz, E. L., Meuer, S. C., Fitzgerald, K. A., Hussey, R. E., Hogdon, J. C., Acuto, O. and Schlossman, S. F., *Proc. Natl. Acad. Sci. USA* 1983 *80* 4104.
9 Chang, T. W., Kung, P. C., Gingras, P. and Goldstein, G., *Proc. Natl. Acad. Sci. USA* 1981 *78* 1805.
10 Spits, H., Borst, J., Terhorst, C. and De Vries, J. E., *J. Immunol.* 1982 *129* 1563.
11 Meuer, S. C., Hussey, R. E., Hogdon, J. C., Hercend, T., Schlossman, S. F. and Reinherz, E. L., *Science* 1982 *218* 471.
12 Tsoukas, C. D., Carson, D. A., Foray, S. and Vaughan, J. H., *J. Immunol.* 1982 *129* 1421.
13 Landegren, U., Ramsted, U., Axberg, I., Ullberg, M., Jondal, M. and Wigzell, H., *J. Exp. Med.* 1982 *155* 1579.
14 Spits, H., Yssel, H., Terhorst, C. and De Vries, J. E., *J. Immunol.* 1982 *128* 95.
15 Breuning, M. H., Spits, H., Ivanyi, P. and De Vries, J. F., *Human Immunol.* 1984 *9* 231.
16 Spits, H., Breuning, M. H., Ivanyi, P., Russo, C. and De Vries, J. E., *Immunogenetics* 1982 *16* 503.
17 Spits, H., Borst, J., Giphart, M., Coligan, J., Terhorst, C. and De Vries, J. E., *Eur. J. Immunol.* 1984 *14* 299.
18 Ledbetter, J. A., Evans, R. L., Lipinski, M., Cunningham-Rudles, C., Good, R. A. and Herzenberg, L. A., *J. Exp. Med.* 1981 *153* 310.
19 Spits, H., Keizer, G., Borst, J., Terhorst, C., Hekman, A. and De Vries, J. E., *Hybridoma* 1984 *2* 423.
20 De Vries, J. E. and Spits, H., *J. Immunol.* 1984 *132* 510.
21 Barnstable, C. J., Bodmer, W. F., Brown, G., Galfre, G., Milstein, C., Williams, A. F. and Ziegler, A., *Cell* 1978 *14* 9.
22 Springer, T. A., Davignon, D., Ho, M. K., Kurzinger, K., Martz, E. and Sanchez-Madrid, F., *Immunol. Rev.* 1982 *68* 111.
23 Berke, G., *Prog. Allergy* 1980 *27* 69.
24 Acha-Orbea, H., Groscurth, P., Lang, R., Stitz, L. and Hengartner, H., *J. Immunol.* 1983 *130* 2952.
25 Brooks, C. G., Urdal, D. and Henney, C. S., *Immunol. Rev.* 1983 *72* 43.
26 Simon, M. M., Weltzien, H. U., Buhring, H. J. and Fichmann, K., *Nature* 1984 *308* 367.
27 Pawelec, G., Schneider, E. M. and Wernet, P., *Immunol. Today* 1983 *4* 275.
28 Friedman, S. M., Thompson, G. S. and Principato, M. A., *J. Immunol.* 1982 *129* 2451.
29 Wee, S. L., Chen, L. K., Strassmann, G. and Bach, F. H., *J. Exp. Med.* 1982 *156* 1854.
30 Fleischer, B., *Nature* 1984 *308* 365.
31 Green, W. R., Ballas, Z. K. and Henney, C. S., *J. Immunol.* 1978 *121* 1566.
32 Palacios, R., *Immunol. Rev.* 1982 *63* 73.
33 Wee, S. L. and Bach, F. H., *Human Immunol.* 1984 *9* 175.

# CHAPTER V

**Anti-T3 induced cytotoxicity: the role of target cell Fc-receptors in the lysis of autologous monocytes and the Fc-independent lysis of T3-positive target cells.**

**Transpl. Proc. 1987. 19:428**

**Leeuwenberg, J., S. Lems, P. Capel.**

# Anti-T3 Induced Cytotoxicity: The Role of Target Cell Fc-Receptors in the Lysis of Autologous Monocytes and the Fc-Independent Lysis of T3-Positive Target Cells

J.F.M. Leeuwenberg, S.P.M. Lems, and P.J.A. Capel

WE HAVE SHOWN previously[1] that anti-T3 monoclonal antibodies (MAb), in addition to blocking specific CTL target-cell interaction, are able to induce lysis of nonspecific targets. By using F(ab')2 fragments of anti-T3 MAb, induction of lysis was shown to be Fc-dependent[1,2]; and in the analysis of a panel of different target cell types, only those targets with demonstrable Fc-receptors (FcR) for murine IgG (sub)classes, were lysed by cytotoxic T lymphocytes (CTL) in the presence of anti-T3 MAb of the appropriate subclass.[2] Anti-CD8, anti-CD4, anti-CD7, and anti-HLA are ineffective in this process, indicating that cell-cell contact via FcR bridging of antibody coated CTLs and targets is not sufficient to induce nonspecific lysis. Activation of the lytic machinery of the CTL via T3 crosslinking by FcR on the target cells, therefore, is a prerequisite for this process.

As an approach to the understanding of the effects of anti-T3 MAb in vivo, we show here that in addition to the lysis of FcR-positive cell lines and heterologous monocytes, CTLs are also able to kill autologous monocytes, ie, monocytes from the same individual from whom the CTLs were derived. Furthermore, evidence is produced for an Fc-independent anti-T3-induced lysis. The latter phenomenon was dependent on the use of T3-positive targets, and this suggests a CTL target-cell bridging via the binding of the F(ab')2 parts of the divalent IgG molecule to the T3 antigens on CTL and target.

*From the Division of Nephrology, University of Nijmegen, St Radboud Hospital, Nijmegen, The Netherlands*

*Supported by the Dutch Kidney Foundation.*

*Address reprint requests to J Leeuwenberg, Department of Medicine, Division of Nephrology, St Radboud Hospital, Geert Grooteplein Z.8, 6525 GA Nijmegen, The Netherlands.*



*0041-1345/87/1901-0134$03.00/0*

## MATERIALS AND METHODS

MAb used were WT31 (anti-T cell receptor),[3] WT32 (anti-CD3),[4] WT82 (anti-CD8),[4] WT1 (anti-CD7),[5] and 7-24 (anti-HLA class I common). F(ab')2 fragments of WT32 were prepared as described[1] An IgG2b switch variant of WT31 (IgG1) was obtained by seeding 100 hybridoma cells of WT31 per microwell using a total of ten plates. After six days, culture supernatants were screened for IgG2b antigen activity by an enzyme-linked immunosorbent assay (ELISA), using coated Goat anti-Mouse Ig (Cappel) and Goat anti-Mouse IgG2b peroxidase (Nordic Pharmaceuticals, Quebec) Positive wells were cloned twice and analyzed for anti-T3 activity.

Cell lines used were the B cell lines: SB, Daudi, and Raji; the T cell lines: CEM, Jurkat, and HPB-all, the erythromyeloid cell line K562; and the monocytic cell lines U937 and HL60 Monocytes were obtained by elutriation of peripheral blood mononuclear cells.[6] Monocytes from an individual whose T lymphocytes respond to anti-T3 MAb of the IgG1 subclass in mitogenesis are indicated as responder (mono R); monocytes unable to support mitogenesis by IgG1 anti-T3 are indicated as nonresponder (mono NR)[7,8]

CTLs, specific for the cell line SB, were prepared and assayed as described previously[1] FcR expression on target cells was determined in immunofluorescence using heat-aggregated mouse IgG subclasses as described[1,2]

## RESULTS

MAb anti-T3 inhibit the specific lysis of CTLs but are also able to induce the lysis of nonspecific targets. As described previously,[1] $FcR^+$ target cells are susceptible to this phenomenon. In an analysis of the susceptibility to anti-T3-induced lysis of a panel of different target cell types, CTLs were tested for their ability to lyse the $FcR^-$ T cell lines CEM, Jurkat, and HPB-all. It was observed that the $T3^+$ lines Jurkat and HPB-all were lysed, whereas the $T3^-$ cell line CEM was not (data not shown). Optimal lysis of Jurkat and HPB-

all was induced by anti-T3 MAb of either the IgG1, IgG2a, or IgG2b subclass at a concentration of 500 ng/ml, whereas MAb anti-CD8, CD7, or HLA were ineffective Because Jurkat and HPB-all have no detectable FcR for mouse IgG, these results suggested an Fc-independent, T3-dependent mechanism of anti-T3-induced lysis, which was investigated further by using F(ab')2 fragments of anti-T3 MAb WT32 and inhibition studies with human serum

As shown in Fig 1, F(ab')2 fragments of WT32 were ineffective in the induction of lysis of T3 target cells, regardless of their FcR expression By contrast, the $T3^+$ target cells Jurkat and HPB-all were shown to be lysed effectively In a second set of experiments it was investigated whether the lysis of $T3^+$ target cells by intact WT32 could be inhibited by Human Serum (HS) as a source of human IgG As is shown in Fig 2, lysis of $FcR^+$, $T3^-$ targets was blocked efficiently by 10% HS, as also observed previously,[2] whereas the lysis of Jurkat and HPB-all either was not or was only slightly affected

These results show that the $T3^+$ cells are lysed in an Fc-independent way We conclude,

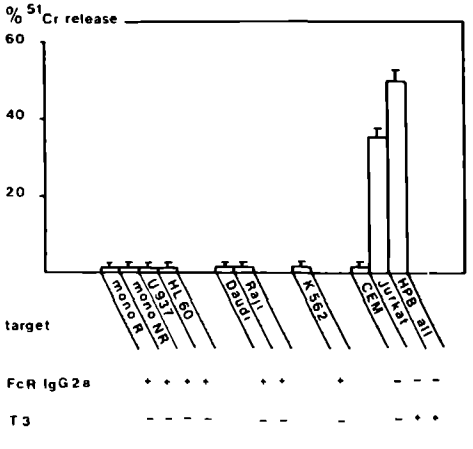


**Fig 1 Induction of nonspecific cytotoxicity by F(ab')2 fragments (500 ng/ml) of WT32 (anti-T3 IgG2a) in relation to the expression of FcR for mouse IgG2a or T3 on the various target cells by CTLs specific for the cell line SB at an effector target ratio of 30/1**

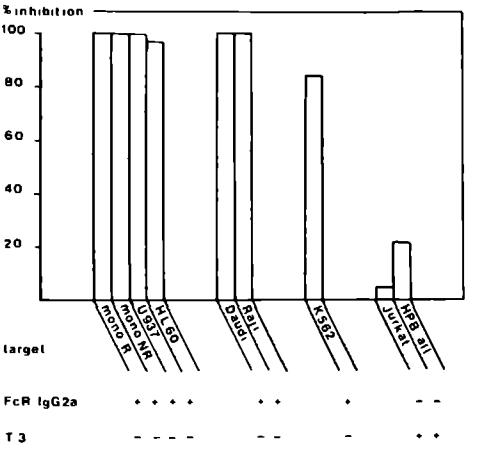


**Fig 2 Inhibition of nonspecific cytotoxicity induced by 30 ng/ml WT32 (anti-T3 IgG2a) by 10% heat-inactivated human serum, in relation to the expression of FcR for mouse IgG2a or T3 on the various target cells by CTL specific for the cell line SB at an effector target ratio of 30/1**

therefore, that an effective bridging of CTL and target can also occur via the T3 complex on CTL and target Consequently, it may be expected that CTLs will lyse themselves in the presence of anti-T3 Indeed, this kind of CTL cannibalism was recently reported by Schrezenmeier et al[9] using a CTL clone However, when we incubated $^{51}Cr$-labeled CTLs with anti-T3, we found no evidence for CTL-CTL kill, using four different CTL lines with various concentrations of anti-T3 (data not shown) Therefore, we investigated whether our CTL lines were able to kill autologous targets per se in an analysis of the susceptibility to lysis of autologous monocytes, ie, monocytes from the same individuals as the CTLs were derived from, as compared to the lysis of heterologous monocytes, ie, from a different individual

In Fig 3 results are shown of an experiment in which two CTL lines were tested with either autologous or heterologous monocytes as targets in the presence of anti-T3 of different subclasses and F(ab')2 fragments of MAb anti-T3 IgG2a The CTLs, raised

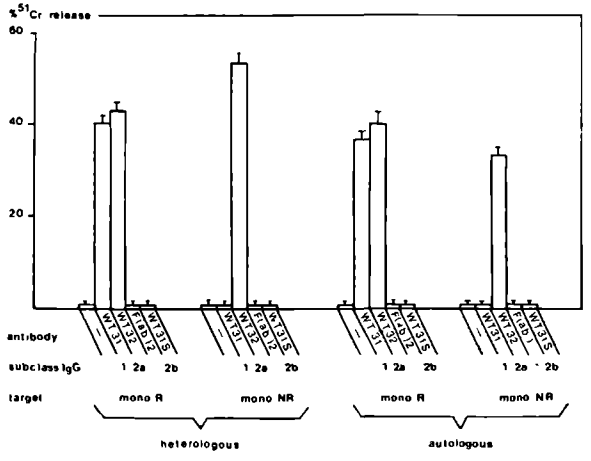


**Fig 3. Induction of nonspecific lysis of heterologous or autologous human monocytes by anti-Ti/T3 antibodies of different subclasses using CTL specific for the cell line SB at an effector target ratio of 30/1. Responder monocytes (non-R) express FcR for murine IgG1 and IgG2a, and nonresponder monocytes (mono NR) only express FcR for murine IgG2a.**

against the B cell line SB, originated from an individual responding to anti-T3 IgG1 in mitogenesis (mono R) and from another unable to respond to anti-T3 IgG1 (mono NR). As is shown, no major differences were observed in the lysis of the autologous *v* the heterologous monocyte targets with both CTL lines.

It is also demonstrated that the findings in mitogenesis are in concordance with the data of anti-T3-induced lysis. Monocytes of a responder (R) were killed by anti-T3 IgG1 as well as IgG2a, whereas monocytes of the nonresponder(NR) lacking the FcR for mouse IgG1 were only killed in the presence of anti-T3 IgG2a. As expected, F(ab')2 fragments of WT32 were ineffective. Also, the IgG2b switch variant of WT31 was ineffective because both donors were shown to be nonresponders to IgG2b anti-T3-induced mitogenesis.

## DISCUSSION

The anti-T3 dependent induction of nonspecific cytotoxicity requires cell-cell contact and a triggering of the lytic machinery of CTLs. The importance of cell-cell contact was demonstrated earlier by the inhibition of anti-T3-induced lysis by anti LFA–1 antibodies, which prevent cell adhesion.[10] That cell-cell contact alone is not sufficient for the induction of lysis is shown by the ineffectiveness of antibodies directed to CD8, CD7, or HLA. Triggering of the lytic machinery is brought about by the crosslinking of either the T cell receptor or T3, as shown here, or by an interaction with the T11 antigen.[11] As is also shown, crosslinking may occur via FcRs on the target cells, by which this process is Fc-dependent, or by means of T3 on the target cells, by which induced cytotoxicity is Fc-independent. This suggests that no additional second signals or target cell membrane modifications, which are generated by FcR crosslinking on target cells, are required for this phenomenon.

Our results with monocytes show that autologous target cells are killed by anti-T3-induced CTLs. In one of two accompanying papers,[12] our first clinical results are described with anti-T3 MAb WT32. A successful reversion of kidney graft rejection was demonstrated in most patients. After a period of T cell elimination, T cells reappeared in the circulation but without detectable T3. Because modulation of the T3/Ti receptor complex may lead to ineffective T cell function, modulation of the T3 antigen was studied as the subject of the second accompanying report.[13] It may be asked whether in addition

to T cell elimination and modulation of T3 the phenomenon of anti-T3-induced lysis, as described here, contributes to the effectiveness of anti-T3 MAb in the treatment of rejection crises by the lysis of $FcR^+$ or $T3^+$ target cells.

## REFERENCES

1 Leeuwenberg JFM, Spits H, Tax WJM, et al: J Immunol 134 3770, 1985

2 Leeuwenberg JFM, Lems SPM, van de Winkel JGJ, et al (in preparation)

3 Spits H, Borst J, Tax WJM, et al: J Immunol 135:1922, 1985

4. Tax WJM, Leeuwenberg JFM, Willems HM, et al: In Bernard A, Boumsell L, Dausset J, et al (eds): Leucocyte Typing. Berlin, Springer-Verlag, 1984, p 721

5 Tax WJM, Tidman N, Janossy G, et al: Clin Exp Immunol 55:427, 1984

6 De Mulder PHM, Wessels JMC, Rosenbrand DA, et al J Immunol Methods 47·31, 1981

7 Tax WJM, Willems HW, Reekers PPM, et al· Nature 304·445, 1983

8. Tax WJM, Hermes FFM, Willems HW, et al: J Immunol 133 1185, 1984

9 Schrezenmeier H, Kurrle R, Wagner H, et al: Eur J Immunol 15 1019, 1985

10 Spits H, Yssel H, Leeuwenberg JFM, et al: Eur J Immunol 15:88, 1985

11 Siliciano RF, Pratt JC, Schmidt RE, et al Nature 317 428, 1985

12 Tax WJM, vd Heijden HMW, Willems HW, et al· Transplant Proc (this issue)

13 Tax WJM, vd Heijden HMW, Capel PJA, et al Transplant Proc (this issue)

# CHAPTER VI

**The role of Fc-receptors in the anti-CD3 induced nonspecific cytotoxicity: specificity of human Fc-receptors for murine IgG subclasses.**

**Submitted for publication**

**Leeuwenberg, J., S. Lems, J. van de Winkel, A. Vlug, P. Capel.**

**SUMMARY**

Addition of monoclonal antibodies (MAb) directed against CD3 or against the T cell receptor (TCR) to cytotoxic T lymphocytes (CTL) can result in a nonspecific lysis of third party target cells. Two types of target cells are susceptible to this type of lysis; Fc receptor (FcR) positive cells and CD3 positive cells. FcR positive cells are only lysed by anti-CD3 MAb of a given subclass when they express an FcR with a reactivity for that particular subclass. This process is Fc dependent, because 1) complete concordance was found between FcR expression on target cells, as determined by binding of purified murine immunoglobulin G (mIgG) subclass aggregates, and their susceptibility to lysis by anti-CD3 MAb of the corresponding subclass and 2) the lack of reactivity of F(ab')2 fragments of anti-CD3, provided the target cells did not carry the CD3 antigen. On the other hand, when target cells express the CD3 antigen themselves, F(ab')2 fragments of anti-CD3 also induced lysis. These findings indicate, that anti-CD3 MAb can effect crosslinking of effector and target cells via CD3-FcR or CD3-CD3 bridges and combined with activation of the CTL via the CD3 complex, will induce nonspecific lysis.

On the basis of this phenomenon and using CD3 negative target cells, the interaction of human FcR with mIgG subclasses was analysed. Different FcR expression patterns were observed. K562, Daudi, and Raji reacted with mIgG1, mIgG2a and mIgG2b; U937, HL60, and responder monocytes (mono R; able to support T cell proliferation induced with anti-CD3 MAb of IgG1 subclass) reacted with mIgG1 and mIgG2a; non responder monocytes (mono NR) reacted only with mIgG2a.

The crossreactivity of the various FcR was determined by inhibition of lysis by aggregates of purified mIgG subclasses, or monomeric human immunoglobulin G (hIgG) subclasses. The FcR for mIgG1 was inhibited by aggregated mIgG1, and less efficiently by aggregated mIgG2a, but not by monomeric hIgG subclasses. The FcR for mIgG2a on monocytes, and on B cells also the FcR for mIgG2b, was inhibited by aggregated mIgG2a, hIgG1 and hIgG3. Surprisingly, however, the FcR for mIgG2a was blocked by aggregated mIgG1 on Daudi and Raji cells, but not on monocytes. These results demonstrate that: 1) the functional FcR expression for the various mIgG isotypes varies on different cell types; 2) the FcR for mIgG2a on monocytes which binds hIgG1 and hIgG3 is functionally different from the FcR for mIgG2a on B cells, suggesting a heterogeneity of this receptor.

## INTRODUCTION

The CD3/TCR complex on T cells plays an important role in T cell function (1). MAb directed against this complex can have apparently opposite effects. Anti-CD3 MAb have inhibitory effects, such as the inhibition of specific cytotoxicity or the blocking of the proliferation of T cells induced by mitogenic stimuli (2, 3). By contrast, binding of MAb to the CD3/TCR complex can also result in triggering of the T cell in an antigen-like fashion, such as the induction of mitogenesis (4) and, as we described recently (5,6), the induction of nonspecific cytotoxicity. Addition of anti-CD3 MAb to MHC restricted CTL resulted in the lysis of third party cells such as the cell lines Daudi, Raji, and K562 in an MHC unrestricted way. Besides anti-CD3, anti-clonotypic or anti-TCR antibodies can also induce nonspecific lysis (7). In a similar way anti-CD3 or anti-clonotype producing hybrids become target for CTL by crosslinking CD3 or TCR via their own antibodies (8, 9). Heteroaggregates of antibodies and anti-CD3 antibodies can induce lysis of cells reacting with the antibody, which is conjugated with anti-CD3 (10, 11, 12).It has also been established that activation of CTL by means of the CD2 glycoprotein instead of via the CD3/TCR complex, induces the same phenomenon (13). This phenomenon can be used for the analysis of various processes, e.g. investigations on the molecular aspects of the mechanism of CTL-target cell destruction, the study of network relationships between T-cells and anti-idiotypic antibodies, and it may eventually be applied for the in vivo treatment of neoplasms by using heterodimers of anti-CD3 and anti-target cell antibodies (11). In our original report we observed that anti-CD3 induced cytotoxicity is an Fc dependent process (5), occurring via the interaction of the Fc-part of anti-CD3 with the FcR on the target cell membrane. Hence, we have used this phenomenon as a novel technique in the analysis of Fc receptors for mIgG (sub)classes on a number of human cell lines amongst which human B cell lines, monocytic cell lines and normal human monocytes. The data demonstrate that FcR for mIgG on human leukocytes are not only heterogeneous in expression, but also heterogeneous in their specificity. It is shown that the expression of a particular FcR varied not only from one cell type to another, but that the specificity of a given receptor on a certain cell type, e.g. the FcR for mIgG2a on B cells, can be functionally different from the corresponding FcR for mIgG2a on monocytes.

**Preparation of lymphocytes**

Peripheral blood mononuclear leukocytes (PBL) of normal healthy donors were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation (14).

**Antibodies and Ig preparations**

Anti-TCR MAb used was WT31 (mIgG1,15). Anti-CD3 MAb used were WT32 (mIgG2a, 16), Leu-4 (mIgG1, Becton Dickinson, Sunneyvale, CA), and SPV-T3a (mIgG2b, 17). For inhibition and fluorescence studies we used MAb PA1, 2-2-1 and 3-1-3-1. PA1 (mIgG1) is an anti-idiotypic MAb, raised against the leukocytes from a patient suffering from a B-CLL (18). MAb 2-2-1 (mIgG2a), and 3-1-3-1 (mIgG2b) were raised against BALB.K spleen cells and reacted with class II and class I $H-2^k$ antigens respectively (19). These antibodies were isolated by Protein A Sepharose chromatography as described by Ey et al. (20). Subsequently, residual IgG and other Ig contaminations were removed by affinity chromatography on Sepharose-4B (Pharmacia, Uppsala, Sweden) columns, coupled with (sub)class specific antisera (Meloy Labs, Springfield, VA, and Litton Bionetics, Kensington, MD) directed against impurities as described (21). The purified IgG preparations were not contaminated (<0.002 mg/ml) by other Ig (sub)classes as judged by gel diffusion analysis with isotype-specific immune sera. Pure human IgG subclass proteins were obtained from the sera of patients with multiple myeloma. They were isolated by $(NH_4)_2SO_4$ precipitation, chromatography on Sephadex DEAE-A50 (Pharmacia) and, if necessary, affinity chromatography on Sepharose columns coupled with Protein A (Pharmacia) or anti-IgG subclass antisera (CLB Amsterdam, The Netherlands). The purity of the isolated proteins were checked by gel precipitation techniques (Mancini, Ouchterlony) and haemagglutination inhibition. The percentage of IgG subclass contamination was less than 1% (22). F(ab')2 fragments of WT32 antibodies were prepared as described previously (5). The percentage of intact IgG was less than 0.03% as assessed in a radio-immunoassay.

**Cell lines**

The cell lines used in this study were the B cell lines SB, Daudi, Raji and RPMI 1788; the T cell lines CEM, Jurkat, and HPB-all; the null cell line REH; the monocytic cell lines HL60 an U937; and the erythromyeloid cell line K562. Monocytes were obtained by elutriation of peripheral blood mononuclear cells as described previously (23).In previous reports from our laboratory on the mitogenic effect of anti-CD3 MAb, a polymorphism was described in the mitogenesis by

anti-CD3 of the mIgG1 (24) and mIgG2b (25) subclass. Individuals, able respectively unable to respond to anti-CD3 MAb of the mIgG1 subclass (70% and 30% of healthy individuals) were indicated as responders (R) and non-responders (NR).

**Cloning of Raji cells**

To obtain cells positive and negative in the expresssion of FcR for mIgG2a cells, Raji cells were cloned in flatbottom microtiter plates (Costar), 1 cell/well, and grown in RPMI 1640, supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 µg/ml gentamycin, and 10% heat-inactivated fetal calf serum. After four weeks samples of the growing clones were tested in immunofluorescence for the presence or absence of FcR for mIgG2a with heat-aggregated (10 min, 63°C) mIgG2a, purified by protein A Sepharose (Pharmacia, Uppsala, Sweden) chromatography.

**Generation and assay of CTL.**

PBL at $10^6$/ml were mixed with 2000 rad-irradiated SB cells ($10^5$ cells/ml) in serum-free Iscove's medium supplemented with 2 x $10^{-5}$ M ethanolamin (Merck, Darmstadt, FRG), 35 µg transferrin, 5 µg/ml insulin, and 2.5 mg/ml bovine serum albumin (BSA; all three from Sigma, St.Louis, MO), 1% (v/v) of a dispersion of lipids extracted from human plasma (17) and 50 µg gentamycin. After 6 days culturing in 24 well plates (Costar) at 37°C in the presence of 5% CO2 the cells were washed and fresh medium was added. After 10 days the cells ($10^6$/ml) were restimulated with the irradiated original stimulator cell ($10^5$/ml) in the presence of irradiated PBL ($10^6$/ml) as a feeder layer. Five days after the second stimulation, the cells were pooled and frozen. Ampoules, containing 4 x $10^7$ cells were stored in liquid nitrogen.

Cytotoxicity was assessed by the standard $^{51}Cr$ release assay. In a total volume of 150 µl, different concentrations of effector cells were incubated in triplicate in the presence or absence of MAb in conical-bottom wells (Titertek; Flow Laboratories) before being mixed with $^{51}Cr$ labeled target cells ($10^4$ cells per 50 µl per well) in RPMI 1640 supplemented with 0.25% BSA. After a 3-min 50 x g centrifugation, the plates were incubated for 3 hr at 37°C followed by centrifugation at 550 x G for 10 min. 100 µl of the supernatants were removed from each well and counted in a gamma counter. The percentage of specific $^{51}Cr$ release was calculated by the following formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release in saponin} - \text{spontaneous release}} \times 100$$

The results are expressed as the means $\pm$ standard deviation of

triplicate experiments.

**Inhibition studies**

Inhibition studies with IgG (murine or human) and with human serum (a pool from 20 normal donors) were performed by preincubation of 50 µl of effector cells and 50 µl of $^{51}Cr$ target cells with 50 µl murine or human IgG, during 15 min, before the addition of anti-CD3 MAb. The concentration of anti-CD3 MAb used in these inhibition studies were chosen such, that the level of induced lysis in the absence of inhibitor was 80 - 100 % of maximal induced lysis.

**Detection of Fc receptors**

Cells (4 x $10^5$) were incubated with heat-aggregated (10 min 60°C) purified mIgG subclasses for 30 min at 4°C in a final volume of 100 µl. After washing three times with phosphate-buffered saline containing 0.25% BSA and 0.05% NaN3, the cells were incubated for 30 min at 4°C with 50 µl of 1/30-diluted fluorescein isothiocyanate labeled sheep anti-mouse Ig antibodies (Cappel), preabsorbed with Sepharose-coupled human Ig to remove cross-reactive antibodies.

## RESULTS

### Fc-dependency of anti-CD3 induced nonspecific cytotoxicity

In order to extend our previous observations on the Fc-dependency of anti-CD3 induced lysis with a mIgG2a MAb (WT32, anti-CD3), we analyzed whether the induction with a mIgG1 MAb (Leu-4, anti-CD3) was similarly Fc-dependent. CTL specific for the cell line SB were preincubated with various concentrations of Leu-4, WT32 , or their F(ab')2 fragments before adding $^{51}Cr$ labeled Daudi cells as targets. As shown in Fig.1, intact mIgG1 MAb (Panel A) as well as intact mIgG2a MAb (Panel B) induced lysis in a dose dependent way, whereas their F(ab')2 fragments did not.

To investigate whether target cell Fc-receptors were involved in this process, a panel of different target cells was tested for the presence of Fc receptors in relation to their susceptibility to nonspecific lysis. Susceptibility to lysis was determined using dilution series of anti-CD3 MAb. Fig. 1 may be taken as an example of such an analysis, and the overall results are listed in Table I.

FcR expression for the different subclasses of mIgG was determined on the target cells by immunofluorescence (IF) using heat-aggregated purified mIgG subclasses. The results as shown in Table I demonstrate that target cells without detectable FcR expression were not lysed by

Table I.

Comparison of anti-CD3 induced lysis with FcR specificity.

| target cell | mIgG1 | | mIgG2a | | mIgG2b | |
|---|---|---|---|---|---|---|
| | lysis[a] | FcR[b] | lysis | FcR | lysis | FcR |
| monocytic cells: | | | | | | |
| U937 | ++ | + | +++ | + | - | - |
| HL60 | + | + | +++ | + | - | - |
| mono R | ++ | + | +++ | + | - | - |
| mono NR | - | - | +++ | + | - | - |
| B cell lines: | | | | | | |
| Daudi[c] | ++ | + | + | + | + | + |
| Raji[c] | + | + | + | + | + | + |
| RPMI 1788 | - | - | - | - | - | - |
| SB | - | - | - | - | - | - |
| erythromyeloid cell line: | | | | | | |
| K562[c] | +++ | + | ++ | + | ++ | + |
| T cell line: | | | | | | |
| CEM | - | - | - | - | - | - |
| null cell line: | | | | | | |
| REH | - | - | - | - | - | - |

[a] Nonspecific lysis by CTL in the presence of WT31 (anti-TCR, mIgG1, WT32 anti-CD3, mIgG2a), and SPV-T3a (anti-CD3, mIgG2b), reaching plateau value in a range of 200-500 ng anti-CD3 MAb/ml (+); 50-200 ng/ml (++); 10-50 ng/ml (+++).

[b] Immunofluorescence with mIgG aggregates of different subclasses to detect mFcR; positive signal at a concentration of 50 µg/ml aggregate: (+).

[c] No cytolysis of Daudi, Raji, and K562 was observed in the absence of MAb.

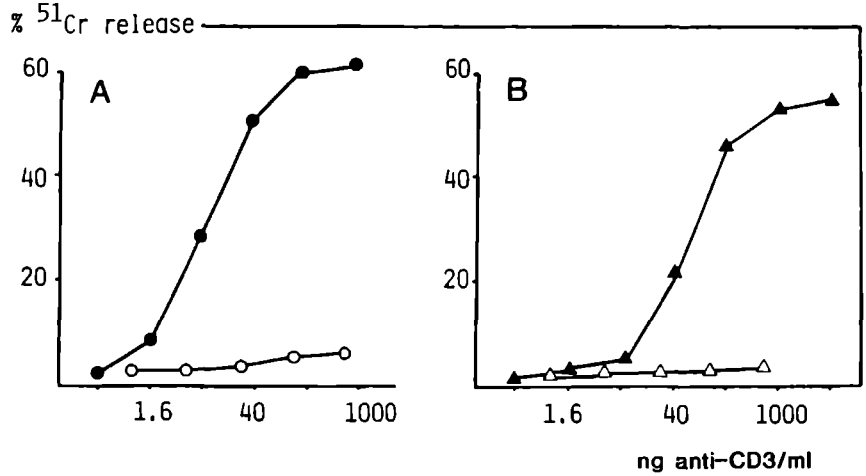


Fig.1. Induction of nonspecific lysis of Daudi cells by anti-CD3 MAb of the mIgG1 (Panel A) and the mIgG2a (Panel B) subclass, either intact (closed symbols) or their F(ab')2 fragments (open symbols). The target to effector ratio was 1 10.

CTL in the presence of anti-CD3 MAb, and that only those target cells which were positive for a particular subclass in the fluorescence assay, were lysed by anti-CD3 MAb of that particular subclass. In addition, the results obtained with monocytes agreed completely with the findings of previous mitogenesis experiments. Responder-type monocytes (mono R) reacted with mIgG1 aggregates in IF, and were lysed by mIgG1 anti-CD3 MAb, whereas non-responder monocytes (mono NR) were not lysed by mIgG1 anti-CD3. Similarly, the monocytes from both types of donors were of non-responder type for the mitogenic effect of mIgG2b anti-CD3 MAb (data not shown), and were also not lysed by CTL in the presence of this MAb.

In order to further study the close relationship between the FcR expression on the target cells and their susceptibility to lysis, we subcloned the cell line Raji and selected cells with or without FcR for mIgG2a. Subsequently, these two sub-lines were tested with a mIgG2a MAb anti-CD3, and the results in Fig. 2 show, that only the Raji cells expressing FcR for mIgG2a, were lysed.

**Fc independent lysis of CD3 positive target cells**

Although the above presented data clearly show an Fc dependency of the anti-CD3 induced lysis, also an Fc independent pathway exists. When various target cells, monocytes responder and nonresponder, U937,

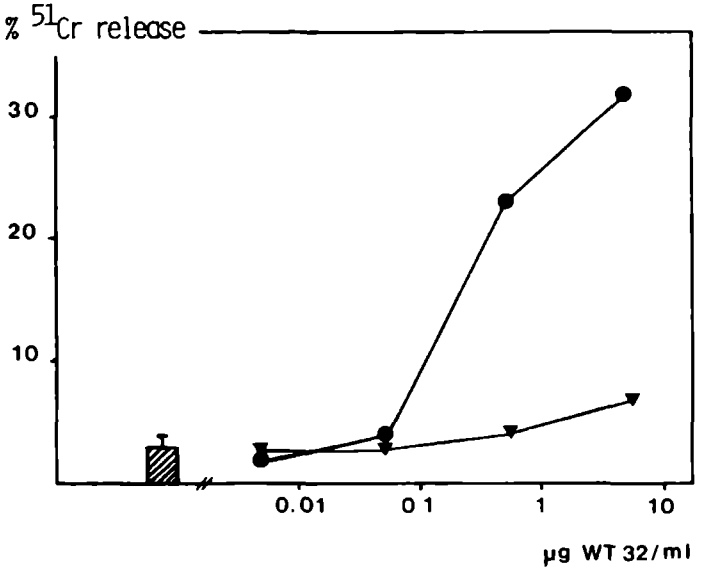


Fig 2 Lysis of cloned Raji cells, either aggregated mIgG2a positive (●–●) or negative (▼–▼) in immunofluorescence, by nonspecific CTL in the presence or absence of WT32 (anti-CD3 MAb, mIgG2a) The shaded bar represents the percent of lysis of Raji cells without addition of antibody The target to effector ratio was 1 10

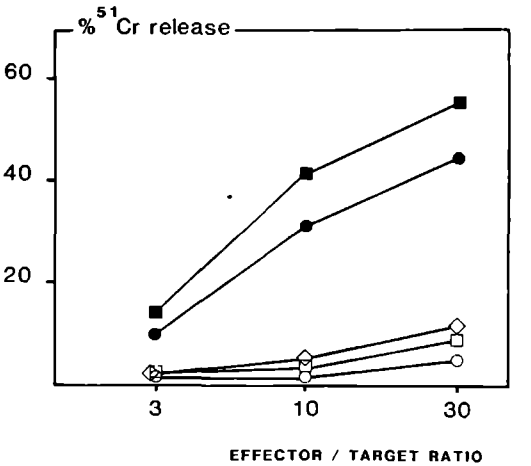


Fig 3 Induction of lysis of CD3-positive cells (Jurkat ■–■, HPB-all ●–●) and CD3-negative cells (K562 and CEM◇–◇, Daudi and Raji □–□, monocytes K, U937, and HL60 ○–○) by F(ab')2 fragments of WT32 (anti-CD3, mIgG2a) at a final concentration of 40 ng ml

HL60, Daudi, Raji, K562, CEM, Jurkat, and HPB-all were incubated with 40 ng/ml F(ab')2 fragments of WT32 and subsequently tested for lysis using SB-specific CTL, only the CD3 positive target cells Jurkat and HPB-all were lysed as is shown in Fig. 3.

**Inhibition of anti-CD3 induced lysis by murine IgG aggregates**

Having demonstrated the heterogeneous expression of FcR for the various mIgG subclasses on various CD3 negative cell types (Table I), we investigated the specificity of different FcR using the inhibition of anti-CD3 induced lysis by mIgG subclass aggregates. CTL and target cells were mixed and preincubated with aggregates of different subclasses of mIgG at various concentrations, before adding anti-CD3 MAb. In Fig. 4 a representative experiment out of a sery of four experiments is shown of the induction of lysis by anti-CD3 mIgG1 of Daudi cells and of human responder-type monocytes as targets. The FcR for mIgG1 was blocked by mIgG1 aggregates on both cell types. This receptor, however, was also blocked, although somewhat less efficient, by mIgG2a aggregates.

A different pattern was observed with the FcR for mIgG2a. On all cell types this receptor was blocked by mIgG2a aggregates. This receptor, however, was also blocked by mIgG1 aggregates on Daudi cells, but not on monocytes (Fig. 5). Lysis of HL60 an U937 by mIgG2a anti-CD3 MAb was also not inhibited by mIgG1 aggregates (data not shown). When monomeric mIgG2a was used as blocking agent, the FcR for mIgG2a was blocked on monocytes, but not on Daudi cells (data not shown). These results indicate that the FcR for mIgG2a on monocytes and monocytic cell lines differs functionally from the mIgG2a FcR on Daudi cells.

The FcR for mIgG2b was investigated on Daudi cells. Aggregates of mIgG1 and mIgG2a blocked this receptor on Daudi cells, indicating an extensive cross-reactivity of this FcR (data not shown).

**Inhibition by human myeloma IgG**

In an attempt to delineate the natural ligands of FcR for mIgG, inhibition studies were performed with human myeloma IgG. Monomeric hIgG1, hIgG2, hIgG3, and hIgG4 were tested in different concentrations with human monocytes and Daudi cells as targets. As is shown in Table II, the FcR for mIgG2a was inhibited by hIgG1 and hIgG3 on both monocytes and Daudi cells, whereas the FcR for mIgG2b on Daudi cells behaved essentially the same. The FcR for mIgG2b was absent on the monocytes tested. However, none of the human myeloma's was able to block the FcR for mIgG1 on Daudi cells, and only a slight inhibition was observed on monocytes. The overall results of the inhibition

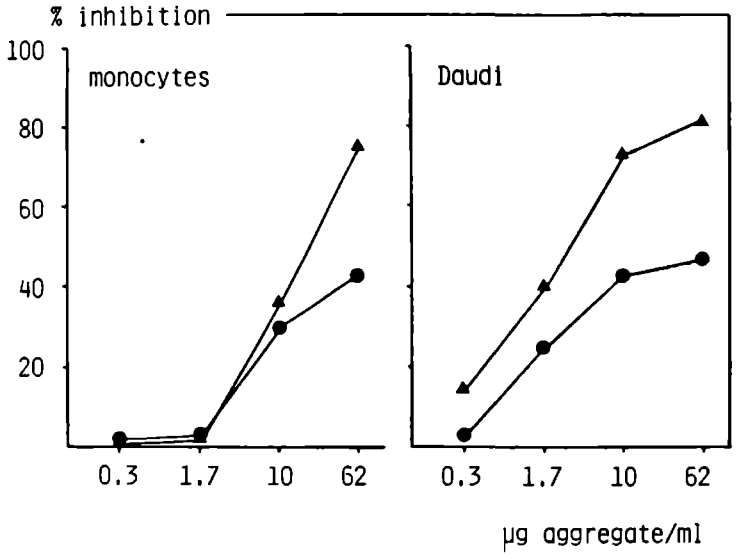


Fig 4 Inhibition of WT31 (anti-TCR, mIgG1) induced lysis by murine aggregates of the IgG1 (▲-▲) subclass and of the IgG2a (●-●) subclass The target to effector ratio was 1 10

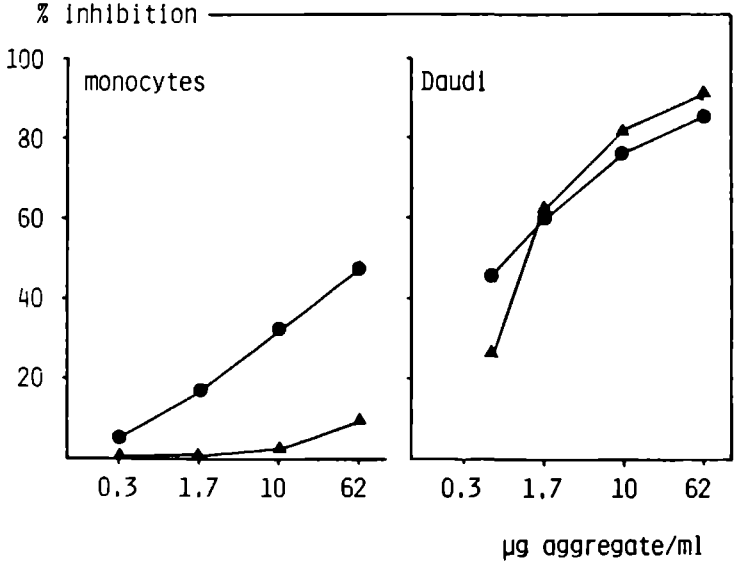


Fig 5 Inhibition of WT32 (anti-CD3, mIgG2a) induced lysis by murine aggregates of the IgG1 (▲-▲) subclass and of the IgG2a (●-●) subclass The target to effector ratio was 1 10

Table II.

Effect of human myeloma IgG on the lysis of Daudi cells and monocytes induced by MAb anti-CD3 of different subclasses.

| human myeloma (mg/ml)[b] | | % inhibition of lysis[a] | | | | |
|---|---|---|---|---|---|---|
| | | Daudi | | | monocytes | |
| | | mIgG1[c] | mIgG2a[d] | mIgG2b[e] | IgG1[c] | mIgG2a[d] |
| IgG1 | 1.250 | 0 | 90 | 70 | 10 | 85 |
| | 0.250 | 0 | 80 | 60 | 5 | 75 |
| | 0.050 | 0 | 40 | 40 | 0 | 55 |
| IgG2 | 0.625 | 0 | 5 | 0 | 15 | 20 |
| | 0.125 | 0 | 0 | 0 | 0 | 5 |
| | 0.025 | 0 | 0 | 0 | 0 | 0 |
| IgG3 | 1.250 | 15 | 80 | 80 | 35 | 100 |
| | 0.250 | 5 | 25 | 40 | 15 | 95 |
| | 0.050 | 0 | 10 | 20 | 5 | 75 |
| IgG4 | 0.125 | 0 | 0 | 0 | 5 | 20 |
| | 0.025 | 0 | 0 | 0 | 10 | 10 |
| | 0.005 | 0 | 0 | 0 | 0 | 0 |

[a] The target to effector ratio was 1:30

[b] Final concentration

[c] WT31 (anti-TCR MAb mIgG1) was added at a final concentration of 25 µg/ml to Daudi cells and of 250 µg/ml to monocytes.

[d] WT32 (anti-CD3 MAb mIgG2a) was added at a final concentration of 50 µg/ml to Daudi cells and to monocytes (R).

[e] SPV-T3a (anti-CD3 MAb mIgG2b) was added at a final concentration of 250 µg/ml to Daudi cells.

experiments with the B-cell line Daudi and monocytes as targets are summarized in Table III.

Table III.
Inhibition of anti-CD3 induced lysis by aggregated and monomeric murine IgG, and monomeric human IgG.

| target cell | FcR | inhibitor: aggregated mIgG1 | aggregated mIgG2a | monomeric mIgG1 | monomeric mIgG2a | monomeric hIgG1 | monomeric hIgG2 | monomeric hIgG3 | monomeric hIgG4 |
|---|---|---|---|---|---|---|---|---|---|
| mono R[a] | mIgG1 | +[b] | + | - | - | - | - | - | |
| | mIgG2a | - | + | + | + | - | + | - | |
| Daudi | mIgG1 | + | + | - | - | - | - | - | |
| | mIgG2a | + | + | - | + | - | + | - | |
| | mIgG2b | + | + | - | + | - | + | - | |

[a] Similar results were obtained using HL60 and U937.
[b] At a concentration of 50 µg/ml aggregated mIgG, 100 µg/ml monomeric mIgG, and 500 µg/ml monomeric hIgG more than 40% inhibition (+).

## DISCUSSION

The Fc dependent anti-CD3 induced cytotoxicity is used to analyze the FcR on monocytes and B cell lines. Addition of anti-CD3 or anti-TCR MAb to CTL results in the lysis of third-party cells such as Daudi, Raji and monocytes. We could demonstrate that this phenomenon is an Fc dependent process: firstly, highly purified F(ab')2 fragments of anti-CD3 were not able to induce lysis; secondly, complete concordance was observed, when the susceptibility to anti-CD3 induced lysis of a panel of different cell types was compared with the reactivity with mIgG aggregates in immunofluorescence; thirdly, a subclone of Raji cells, lacking the FcR for mIgG2a was not lysed by anti-CD3-IgG2a. The proposed mechanism is, that the Fc-part of the anti-CD3, bound to the CD3-complex on the effector cells, binds to the FcR on the target cell. In this way a bridge between the target and the CTL is formed,

resulting in an effective triggering of the lytic machinery of the CTL by crosslinking the CD3-complex.
With the presence of CD3 as well on the target as on the effector cells, a similar type of bridging can occur via the CD3 antigen on the target and effector cell, resulting in an Fc-independent lysis. This mechanism was observed with F(ab')2 fragments of anti-CD3 on CD3 positive target cells.
Although Schrezenmeier et al. (26) failed to show FcR dependent killing of nonspecific target cells by cytotoxic T cell clones in the presence of anti-CD3 MAb, Staerz et al (27) confirmed the Fc dependency in the mouse system with a MAb of mIgG2a subclass directed against the T cell receptor; only those targets, which expressed receptors for mIgG2a were lysed by CTL, incubated with the MAb. Similarly Gullberg et al. (28) demonstrated, also in a mouse system, nonspecific killing of B cells by CTL in the presence of a rabbit-anti-mouse brain antiserum, which could activate T cells. This lysis was inhibited by normal rabbit IgG, which also indicates Fc involvement.
Having established the Fc-dependency of the induction of nonspecific cytotoxicity for CD3 negative target cells, this phenomenon was used as an assay for FcR characterization. The presence or absence of FcR for the different subclasses of mIgG was demonstrated by using anti-CD3 or anti-TCR MAb of different subclasses. With this approach it was shown that Daudi, Raji and K562 cells were positive for mIgG1, mIgG2a, and mIgG2b; monocytes and the monocyte related cell lines U937 and HL60 were positive for mIgG1 and mIgG2a and monocytes non-responsive in the mIgG1-anti-CD3 induced mitogenesis were only positive for mIgG2a.
Our data show, that at least functionally the mIgG1, mIgG2a, and mIgG2b subclasses are handled differently by the various cell types, and that for a given FcR the crossreaction patterns with other subclasses can vary also for the different cell types.
Experiments of anti-CD3 induced proliferation of T cells show a monocyte dependent polymorphism in the handling of mIgG1 and mIgG2b antibodies. This could be ascribed to the presence or absence of functional FcR for these subclasses (24, 25). Monocytes from 70% of the caucasian population display a receptor for mIgG1 and less than 10% a receptor for mIgG2b (25). Not only mIgG1-/mIgG2a+/mIgG2b- individuals are described, but also mIgG1-/mIgG2a+/mIgG2b+ individuals (25). This indicates the existence of three functionally distinct FcR. However, from these data one cannot conclude that these functions are also located in three separate receptor structures. Although three

biochemically different FcR have been described (29) localisation of a function to only one receptor is difficult, due to the crossreactivity of these receptors with other subclasses. Minor variations either in the receptor itself or in the quantitative expression can influence the outcome of a test. The definition of the presence or absence of a receptor will depend on the sensitivity of the assay used. An example of different results obtained with different tests is the polymorphism of the mIgG1 FcR on human monocytes. This polymorphism is clear-cut and genetically determined (25) when measured in anti-CD3 induced proliferation. However, using a very sensitive quantitative rosette assay, this receptor could still be detected on monocytes lacking accessory function in anti-CD3 induced T cell proliferation. This expression, at least functionally, was much less in these individuals (30).
Besides quantitative differences minor variations within the receptor might be responsible for the functional differences as was shown by Anderson et al. (31), who observed differences in isoelectric focussing patterns of purified FcR for mIgG1 between high and low responder monocytes. Our data show that B cell lines display a different crossreactivity pattern in comparison with monocytes for the mIgG2a FcR, when tested with mIgG1 aggregates. From this difference, however, one cannot postulate entirely different receptor types, but qualitative or quantitative differences might explain these results. The crossreaction patterns which can vary per cell type makes that the choice of cells together with the sensitivity of the assay used, are important for the observed specificity of the receptors studied. In this respect the literature is conflicting concerning the presence of mIgG2a FcR on the cell lines Daudi, Raji, and K562. Our data show a functional mIgG2a FcR on these cells, and it has also been reported that mIgG2a inhibit EA rosette formation on K562 cells (32). On the other hand, measuring the function of K562 to induce interleukin-2 release from Jurkat cells in the presence of anti-CD3 MAb, Leu 4 (mIgG1) was active and OKT3 (mIgG2a) was not, suggesting the absence of a mIgG2a FcR (33), and the same was found for Daudi cells (34). Our explanation for these different findings is that these might be due to differences in sensitivity of the assay used. Furthermore, FcR expression on cell lines can vary in time and are dependent on cell cycle (35, 36). We were able to clone Raji cells at the single cell level, and obtained FcR-mIgG2a positive and negative clones. Long term culture of more than four weeks of these clones resulted in the gradual reappearance of the FcR for mIgG2a on the initially FcR-mIgG2a negative clones.

Our data show, that the receptor for mIgG1 does not react with monomeric human IgG, and that this receptor, when present on human cells, crossreacts with mIgG2a. This receptor has recently been characterized as the 40 kD FcR by Anderson et al. (31). The FcR for mIgG2a is present on human monocytes, the cell lines U937 and HL60, and is the high affinity 72 kD FcR (34). This receptor is also present on K562, Daudi, and Raji. On Daudi cells this receptor crossreacted with mIgG1 in contrast to the FcR on monocytes, HL60 and U937 which did not show any reactivity with mIgG1, indicating functional differences of this receptor on various cell types, which suggests heterogeneity of this receptor. This FcR for mIgG2a reacted in all cases with monomeric human IgG1 and IgG3.

The FcR for mIgG2b was only detectable on K562, Daudi and Raji and not on other cell types tested. When present, this receptor had a comparable inhibition profile with other subclasses as the FcR for mIgG2a on these cells. The different cellular expression in comparison with the FcR for mIgG2a could suggest a distinct receptor for mIgG2b. On the other hand the inhibition profile was similar to the FcR for mIgG2a. Our preliminary data obtained from comodulation studies, suggest that mIgG2b can be bound to the FcR for mIgG1. Therefore, further studies are necessary before it can be concluded whether the FcR for mIgG2b is a separate receptor, or the observed reactivity is due to a crossreaction of other FcR on these particular cells.

We can conclude, that the phenomenon of anti-CD3 induced cytotoxicity, when using CD3 negative target cells, can be a valuable tool for the further analysis of FcR profiles on cells and their specificity.

Acknowledgments. We thank Dr. W.A. Buurman, and Prof.dr. R.A.P. Koene for their critical reading of the manuscript.

**REFERENCES**

1. Chang, T., P. Kung, S. Gingras, and G. Goldstein. 1981. Does OKT3 monoclonal antibody react with an antigen-recognition structure on human T cells? Proc. Natl. Acad. Sci. USA 78:1805.
2. Platsoucas, C., and R. Good. 1981. Inhibition of specific cell mediated cytotoxicity by monoclonal antibodies to human T cell antigens. Proc. Natl. Acad. Sci. USA 78:4500.
3. Meuer, S., K. Fitzgerald, R. Hussey, J. Hodgdon, S. Schlossman, and E. Reinherz. 1983. Clonotypic structures involved in antigenspecific human T cell function. Relationship to the T3 molecular complex. J. Exp. Med. 157:921.
4. Van Wauwe, J., J. DeMey, and J. Goossens. 1980. OKT3: a monoclonal anti-human lymphocyte antibody with potent mitogenic properties. J. Immunol. 124:2708.
5. Leeuwenberg, J., H. Spits, W. Tax, and P. Capel. 1985. Induction of nonspecific cytotoxicity by monoclonal anti-T3 antibodies. J. Immunol. 134:3770.
6. Spits, H., H. Yssel, J. Leeuwenberg, and J. de Vries. 1985. Antigenspecific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. Eur. J. Immunol. 124:2708.
7. Kranz, D., S. Tonegawa, and H. Eisen. 1984. Attachment of an antireceptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA 81:7922.
8. Hua, C., M. Buferne, and A. Schmitt-Verhulst. 1985. Lysis of hybridoma cells bearing anti-clonotypic surface immunoglobulin by clonotype-expressing alloreactive T cells. Eur. J. Immunol. 15:1029.
9. Hoffman, R., J. Bluestone, O. Leo, and S. Shaw. 1985. Lysis of anti-T3 bearing murine hybridoma cells by human allospecific cytotoxic T cell clones and inhibition of that lysis by anti-T3 and anti-LFA-1 antibodies. J. Immunol. 135:5.
10. Staerz, U., O. Kanagawa, and M. Bevan. 1985. Hybrid antibodies can be target sites for attack by T cells. Nature 314:628.
11. Perez, P., R. Hoffman, S. Shaw, J. Bluestone, and D. Segal. 1985. Specific targeting of cytotoxic T cells by anti-T3 linked to antitarget cell antibody. Nature 316:354.
12. Perez, P., R. Hoffman, J. Titus, and D. Segal. 1986. Specific targeting of human peripheral blood T cells by heteroaggregates containing anti-T3 crosslinked to anti-target cell antibodies. J. Exp. Med. 163:166.
13. Silicano, R., J. Pratt, R. Schmidt, J. Ritz, and E. Reinherz. 1985. Activation of cytolytic T lymphocytes and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature 317:428.
14. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. Clin. Lab. Invest. (Suppl.) 21:77.
15. Spits, H., J. Borst, W. Tax, P. Capel, C. Terhorst, and J. de Vries. 1985. Characteristics of a monoclonal antibody (WT31) that recognizes a common epitope on the human T cell receptor for antigen. J. Immunol. 135:1922.
16. Tax, W., J. Leeuwenberg, H. Willems, P. Capel, and R. Koene. 1984. Monoclonal antibodies reactive with OKT3 antigen or OKT8 antigen. In: Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies. Springer Verlag, Berlin. P. 721.
17. Spits, H., G. Keizer, J. Borst, C. Terhorst, A. Hekman, and J. de Vries. 1983. Characterization of monoclonal antibodies against cell surface molecules associated with cytotoxic activity of natural and activated killer cells and

cloned CTL lines. Hybridoma 2:423.
18. Capel, P , F. Preyers, W. Allebes, and C. Haanen. 1985. Treatment of chronic lymphocytic leukaemia with monoclonal anti-idiotypic antibody. Neth. J. Med. 28:112.
19. Lems, S., W. Tax, P. Capel, and R. Koene. 1983. Immune regulation by monoclonal antibodies: failure to enhance mouse skin allografts. J. Immunol. 131:86.
20. Ey, P., S. Prowse, and C. Jenkin. 1978. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein-A sepharose. Immunochemistry 15:429.
21. Lems, S., T. Rijke-Schilder, P. Capel, and R. Koene. 1984. Similar activities of IgG1 and IgG2 alloantibodies in enhancement of mouse skin grafts and in opsonization. Transplantation 37:291.
22. Klein, F., F. Skvaril, R. Vermeeren, A. Vlug, and W. Duimel. 1985. The quantification of human IgG subclasses in reference preparations. Clin. Chim. Acta 150:119.
23. Mulder, P de, J. Wessels, D. Rosenbrand, J. Smeulders, D. Wagener, and C. Haanen. 1981. Monocyte purification with counterflow centrifugation monitored by continuous flow cytometry. J. Immunol. Methods 47:31.
24. Tax, W., H. Willems, P. Reekers, P. Capel, and R. Koene. 1983. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. Nature 304:445.
25. Tax, W., H. Spits, H. Hermes, H. Willems, P. Capel, and R. Koene. 1985. Polymorphism of human Fc receptors for mouse IgG1 and IgG2b. Transpl. Proc. 17:794.
26. Schrezenmeier, H., R. Kurrle, H. Wagner, and B. Fleischer. 1985. Activation of human T lymphocytes. III. Triggering of bystander cytotoxicity in cytotoxic T cell clones by antibodies against the T3 antigen or by a Calcium ionophore. Eur. J. Immunol. 15:1024.
27. Staerz, U., and M. Bevan. 1985. Cytotoxic T lymphocyte-mediated lysis via the Fc receptor of target cells. Eur. J. Immunol. 15:1172.
28. Gullberg, M., and A. Coutinho. 1985. Activation of Lyt-2+ T cells by antibodies towards brain-associated antigens. II. Antibodydependent induction of "nonspecific" cell-mediated cytotoxicity. Eur. J. Immunol. 15:971.
29. Anderson, C., and R. Looney. 1986. Human leukocyte IgG Fc receptors. Immunol. Today 7:264.
30. Winkel, J. van de, W. Tax, M. van Bruggen, K. van Roozendaal, R. Willems, A. Vlug, P. Capel, and R. Koene. 1987. Characterization of two Fc receptors for mouse immunoglobulins on human monocytes and cell lines. Scand. J. Immunol. 26:663.
31. Anderson, C., D. Ryan, R. Looney, P. Leary. 1987. Structural polymorphism of the human monocyte 40 kD Fc receptor for IgG. J. Immunol. 138:2254.
32. McCool, D., B. Birshtein, and R. Painter. 1985. Structural requirements of immunoglobulin G for binding to the $Fc_\gamma$ receptors of the human tumor cell lines U937, HL60, ML1, and K562. J. Immunol. 135:1975.
33. Landegren, U., J. Andersson, and H. Wigzell. 1985. Analysis of human T lymphocyte activation in a T cell tumor model system. Eur. J. Immunol. 15:308.
34. Looney, R., G. Abraham, and C. Anderson. 1986. Human monocytes and U937 cells bear two distinct Fc receptors for IgG. J. Immunol. 136:1641.
35. Gandour, D., and W. Walker. 1983. Macrophage cell cycling: influence on Fc receptors and antibody-dependent phagocytosis. J. Immunol. 130:1108.
36. Raychandhuri, G., D. McCool, and R. Painter. 1985. Human IgG1 and its Fc fragment bind with different affinities to the Fc receptors on the human U937, HL60 and ML-1 cell lines. Molec. Immunol. 22:1009.

# DISCUSSION AND SUMMARY

This thesis can be divided in two main subjects. Part one describes a study of some MAb against T cell differentiation antigens. The second part deals with the interaction of MAb with human Fc receptors.
The incentive of the study of MAb directed against T cell differentiation antigens was to develop more selective and more effective agents for the immunotherapy of renal transplantation patients, than the conventional polyclonal ATG. An immunosuppressive drug can be used either to prevent an immune reaction, or to suppress an existing immune response. Usually ATG treatment is started when the rejection has been diagnosed, and this will also be the case if MAb is used. Prevention, however, of a response may be preferable in some particular cases.
In Chapter II the characteristics of an anti-pan T cell MAb (WT1) directed against the CD7 antigen, are described. WT1 was prepared by the hybridoma technique after immunization of mice with human thymocytes. It binds to a 40 kD glycoprotein (CD7) (1) present on T lymphocytes, thymocytes, and on most T cell lines. WT1 also reacts with the T lymphocytes of Rhesus monkeys. This property made WT1 suitable to study its immunosuppressive capacities in the Rhesus monkey model. Administration of WT1 to Rhesus monkeys carrying skin allografts resulted in prolonged graft survival. The mechanism by which WT1 was effective, is not clear. WT1 was administered two days prior to transplantation, and the animals received daily injections of WT1 during the following 10 days. This treatment was apparently able to delay the induction of an immune response. However, when applied as immunosuppressive drug during rejection in a human renal transplantation, successful suppression of the rejection process was not obtained. This suggests, that WT1 is not able to influence an ongoing immune reaction, but that this MAb might only affect the initiation phase of an immune response. Whether anti-CD7 MAb will be effective in clinical transplantation, when given prophylactically, is not clear at this time. A preliminary report on the prophylactic use of RFT2 MAb (anti-CD7) in renal transplantation is not very encouraging; two of five patients developed a rejection episode while receiving treatment with RFT2 (2). The binding of MAb directed against CD7 results in a rapid modulation of the antigen in vitro. This process was also observed in vivo during administration of WT1 to patients who had a rejection of their kidney grafts. In these cases, the amount of circulating T cells in the periphery was not affected by the WT1 treatment, but the CD7 antigen was lacking on these cells due to

modulation These data suggest, that although the expression of CD7 is increased on activated T cells, the antigen itself is not involved in the actual effector function of the T cell Thus, the absence of the CD7 antigen from the modulated T cells has no influence on their function. In vitro, anti-CD7 does not interfere with functional assays such as mixed lymphocyte reaction or cytotoxic assays, also indicating that the antigen is not directly functionally involved in T cell activation

The strong modulation of CD7 after binding of anti-CD7, which might be the reason for the in vivo ineffectiveness of WT1 treatment, is on the other hand beneficial when this antibody is conjugated with the Ricin-A chain This immunotoxin is rapidly internalized by CD7 positive cells, proved to be very effective in killing of CD7 positive leukemic cells, and can be used for purging of bone marrow from patients suffering from acute lymphatic T cell leukemia, prior to autologous bone marrow grafting (3)

In contrast to WT1, MAb directed against the CD3 complex interfere in different functional assays, presumably because CD3 is closely associated with the T cell receptor. Foreign antigens initiate an immune response via binding of the T cell receptor resulting in activation of T cells. Anti-CD3 MAb inhibit this activation process, when specific antigen is used as stimulator, the antigen specific cell-mediated lympholysis and the proliferation to soluble antigen are blocked by anti-CD3 MAb (4, 5) On the other hand, anti-CD3 MAb can also mimic antigen by binding to the CD3/T cell receptor complex, and in this way activating T cells in an antigen nonspecific way, such as induction of mitogenesis, release of interferon-gamma, expression of IL2 receptors (6, 7, 8, 9). Furthermore, as described in Chapter III, anti-CD3 MAb induce nonspecific killing of third-party cells by cytotoxic T cells (CTL)

Cell-mediated lympholysis can be divided into several steps (10). Initially, adhesion of CTL to the target cell occurs This phase is antigen-independent and can be blocked, accordingly to the T cell phenotype, by anti-CD8, anti-CD4, anti-CD2, and anti-LFA-1 MAb The adhesion step is followed by the "programming for lysis", after which the lethal hit" will be delivered This process can be inhibited by anti-CD3 MAb. However, as described in Chapter III and the following Chapters, anti-CD3 MAb can also trigger antigen-specific cytotoxic T cells to lyse antigen nonspecific target cells This phenomenon is called anti-CD3 induced cytotoxicity", and was only observed with activated T cells, obtained from a mixed lymphocyte culture (MLC) or with T cell clones Whereas cytotoxic T cells show

nonspecific cytotoxicity, freshly isolated peripheral blood lymphocytes and thymocytes did not show nonspecific cytotoxicity in the presence of anti-CD3 MAb. The lytic capacity is apparently not functionally present in these resting cells, but becomes operational once the "future cytotoxic" T cells are activated by antigen, mitogens or anti-CD3 antibodies.

When a panel of different T cell clones was investigated for their capacity to lyse nonspecific target cells in the presence of anti-CD3 MAb, CD4 positive as well as CD8 positive T cell clones could be induced to nonspecific cytotoxicity by anti-CD3 MAb. Not only antigen specific cytotoxic T cell clones, but also noncytotoxic antigen specific proliferating T cell clones were rendered susceptible to nonspecific lytic activity by anti-CD3 MAb. However, two antigen specific proliferating T cell clones were found, which could not be induced to nonspecific lytic activity, indicating that not all activated T cells per se possess a lytic machinery (Chapter IV)

Induction of nonspecific lysis requires more than only divalent binding of antibody to the CD3 complex, F(ab')2 fragments of anti-CD3 were unable to induce lysis of CD3 negative target cells, whereas intact antibodies did, provided the target cells expressed an FcR for the appropriate murine IgG subclass used in the system When the target cells expressed the CD3 antigen, F(ab')2 fragments of anti-CD3 were also able to induce lysis of these cells (Chapter V and VI). These findings may explain the mechanism by which anti-CD3 induced lysis is effective, anti-CD3 forms a bridge between the target cell and the CTL via either the FcR or the CD3 complex on the target cell and the CD3 complex on the CTL. This bridge formation leads also to crosslinking of the CD3 complex on the CTL, resulting in triggering of the lytic machinery, and subsequently in lysis of the target cell. In this proposed mechanism the CD3 complex plays a pivotal role in signal transduction leading to the activation of the lytic machinery A similar result can be obtained when a combination of anti-CD2 MAb are used, nonspecific lysis can occur in the presence of anti-$T11_2$ and anti-$T11_3$ MAb (11) This is the same combination which can activate T cells, resulting in proliferation and IL2 release (12). When MAb directed against other differentiation antigens were tested, none of them were able to induce cytolytic activity, indicating that only antigens involved in T cell activation deliver the appropriate signal by which the lytic machinery is triggered.

Since FcR expressing cells or CD3 positive cells can be lysed nonspecifically in the presence of anti-CD3 MAb, autolysis of CTL could theoretically also occur In our experiments, however, autolysis

of CTL was not observed It has been described, that CTL per se can be lysed by other CTL, when $CTL_A$ directed against B, and $CTL_B$ directed against C, were mixed, unidirectional killing of $CTL_B$ occurred (13) Kranz and Eisen (14) compared the susceptibility to lysis of CTL and noncytolytic cell lines by either a CTL clone or CTL, derived from an MLC Their results indicated that CTL are more resistant to lysis than the other cell types tested. The susceptibility to lysis appeared to be dependent on the aggressor CTL, the CTL-targets were completely resistant to lysis by some, but not all the CTL, tested as effector. These findings may explain results of other investigators, who described autolysis of CTL in the presence of anti-CD3 MAb (15) Autologous monocytes, however, are not spared from lysis by autologous CTL in the presence of anti-CD3 MAb (Chapter V) The importance of this phenomenon in the in vivo treatment of anti-CD3 MAb as immunosuppressive drug during rejection of allografts has yet to be elucidated. Many cells, including CTL are thought to play a role in the rejection of allografts, e.g. activated macrophages, B cells, and antibody dependent cellular cytotoxic (ADCC) cells These cell types are all gathered in the battlefield of rejection and express FcR. It is possible that not only CD3 positive cells but also FcR positive cells are killed by CTL during treatment with anti-CD3, resulting in removal of various types of immunoreactive cells, which are responsible for the rejection.

The anti-CD3 induced cytotoxicity can be used to study different processes such as the molecular aspects of the mechanism of CTL-target cell destruction, or the analysis of network relationships between T cells and anticlonotypic antibodies A clinical application may be the in vivo treatment of tumors, using heteroaggregates of anti-CD3 and anti-tumor specific antibodies (16) Similarly, heteroconjugates of anti-TCR MAb and MAb specific for influenza A virus proteins may be an approach in anti-viral therapy (17). Since FcR positive target cells are susceptible to the anti-CD3 induced lysis, this phenomenon provides a useful approach for the study and the analysis of FcR
The second part of this thesis deals with the characterization of FcR for murine IgG on human leukocytes (Chapter VI). The anti-CD3 induced lysis occurs via the interaction of the Fc-part of anti-CD3 with the FcR on the target membrane, provided these target cells are CD3 negative. The presence or absence of FcR for a particular (murine) IgG subclass could be investigated using anti-CD3 MAb of different IgG subclasses The FcR expression was studied on human cell lines amongst which human B cell lines, a proerythromyeloid cell line

(k562), monocytic cell lines (HL60 and U937), and normal human monocytes. Different FcR expression patterns were observed, some cell types were reactive with murine IgG1, IgG2a, and IgG2b (B cell lines, k562), others with murine IgG1 and IgG2a (HL60, U937, monocytes), whereas also cells only reactive with murine IgG2a were found (monocytes from circa 30% of the individuals). The specificity of FcR was analyzed by inhibition studies of the FcR with aggregates of purified murine IgG subclasses, and monomeric human IgG subclasses on human monocytes and the B cell line Daudi. The FcR for murine IgG1 can be blocked by murine IgG2a, but not by human IgG subclasses. The FcR for murine IgG2a, however, can be blocked by human IgG1 and IgG3. Aggregates of murine IgG1 are able to block the receptor for murine IgG2a on Daudi cells, but not the receptor for murine Ig2a on monocytes, suggesting that this receptor is functionally different on different cell types.

In T cell proliferation studies using anti-CD3 MAb it was found that 30% of the caucasian individuals did not respond to anti-CD3 MAb of the IgG1 subclass (18). A polymorphic FcR for murine IgG1 on human monocytes was responsible for this phenomenon. It was concluded that monocytes of some individuals (non responder monocytes) lacked the FcR for murine IgG1. Due to this lack of FcR, no crosslinking of the CD3 complex occurred, which is an essential requirement for T cell proliferation. Also for the FcR for murine IgG2b a polymorphism was observed. mitogenesis could be induced by anti-CD3 of the IgG2b subclass in less than 10% of the caucasian population (19). This polymorphism was also observed in the anti-CD3 induced cytotoxicity; non responder monocytes were not lysed by CTL in the presence of anti-CD3 MAb of the IgG1 subclass. A very sensitive EA rosetting assay, however, could still detect an FcR for murine IgG1 on the non responder monocytes, when very high concentrations of sensitizing antibody were used (20). Similarly, Clement et al. (21) were able to induce mitogenesis in T cells of a non-responding individual using a thousandfold higher concentration anti-CD3 MAb of the IgG1 subclass. These findings indicate that the outcome of the test is dependent on its sensitivity, and in the case of mitogenesis, also on the capacity of the FcR positive accessory cell to deliver the second signal, necessary for proliferation of the T cells (22).

Biochemically, three different FcR for human IgG on human cells have been described: a 72 kD FcR (FcRI), binding human IgG and murine IgG2a and IgG3 is present on human monocytes; a 40 kD FcR (FcRII), is expressed on macrophages, platelets, granulocytes, and B cells and binds murine IgG1 and very poorly human IgG, and an FcR with very low

affinity for human IgG ($FcR_{lo}$) (23), present on neutrophils, eosinophils, macrophages, natural killer cells, killer cells, large granular lymphocytes, and $T_\gamma$ cells. On Daudi cells only the 40 kD FcR was isolated (24). Monocytes, on the other hand, express in circa 70% of the individuals two different FcR; the 72 Kd FcR and the 40 kD receptor. The 40 kD receptor on monocytes has not the same characteristics as the 40 kD receptor on Daudi cells (and on K562 cells), since it binds no murine IgG2b, and has a lower affinity for murine IgG1 than the 40 kD receptor on Daudi cells. These results indicate, that the FcR are not only heterogeneous in expression, but also heterogeneous in their specificity on different cell types.

REFERENCES


1. Link, M., Warnke, R., Finlay, J., Amylon, M., Miller, R., Dilley, J., Levy, R. A single monoclonal antibody identifies T-cell lineage of childhood lymphoid malignancies. Blood 1983; 62:722-728.
2. Raftery, M., Lang, C., Ivory, K., Tidman, N., Sweny, P., Fernando, O., Moorhead, J., Janossy, G. Use of RFT2 (CD7) monoclonal antibody as prophylaxis against renal allograft rejection. Transplant. Proc. 1985; 17:2737-2739.
3. Myers, C., Thorpe, P., Ross. W., Cumber, A., Katz, F., Tax, W., Greaves, M. An immunotoxin with therapeutic potential in T cell leukemia: WT1-ricin A. Blood 1984; 63:1178-1185.
4. Meuer, S., Fitzgerald, K., Hussey, R., Hodgdon, J., Schlossman, S. Reinherz, E. Clonotypic structures involved in antigen specific human T cell function: relationship to the T3 molecular complex. J. Exp. Med. 1983; 157:705-719.
5. Chang, T., Kung, P., Gingras, S., Goldstein, G. Does OKT3 monoclonal antibody react with an antigen-recognition structure on human T cells? Proc. Natl. Acad. Sci. USA 1981; 78:1805-1808.
6. Weiss, J., Daley, J., Hodgdon, J., Reinherz, E. Calcium dependency of antigen-specific (T3-Ti) and alternative (T11) pathways of human T-cell activation. Proc. Natl. Acad Sci. USA 1984; 81:6836-6840.
7. Wauwe, J. van, de Mey, J., Goossens, J. OKT3: a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. J. Immunol. 1980; 124:2708-2713.
8. Tsoukas, C., Landgraf, B., Bentin, J., Valentine, M., Lotz, M., Vaughan, J., Carson, D. Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes. J. Immunol. 1985; 135:1719-1723.
9. Von Wussow, P., Platsoucas, C., Wiranowska-Stewart, M., Stewart, W. Human γ interferon production by leukocytes induced with monoclonal antibodies recognizing T cells. J. Immunol. 1981; 127:1197-1200.
10. Berke, G. Cytotoxic T-lymphocytes how do they function? Immunological Rev. 1983; 5-42.
11. Siliciano, R., Pratt, J., Schmidt, R., Ritz, J., Reinherz, E. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature 1985; 317:428-430.
12. Meuer, S., Hussey, R., Fabbi, M., Fox, D., Acuto, O., Fitzgerald, K., Hodgdon, J., Protentis, J., Schlossman, S., Reinherz, E. An alternative pathway of T-cell activation: a functional role for the 50 KD T11 sheep erythrocyte receptor protein. Cell 1984; 36:897-906.
13. Kuppers, R., and Henney, C. Studies on the mechanism of lymphocyte-mediated cytolysis. IX. Relationships between antigen recognition and lytic expression in killer T cells. J. Immunol. 1977; 118:71-76.
14. Kranz, D., Eisen, H. Resistance of cytotoxic T lymphocytes to lysis by a clone of cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA 1987; 84:3375-3379.
15. Schrezenmeier, H., Kurrle, R., Wagner, H., Fleischer, B. Activation of human T lymphocytes. III. Triggering of bystander cytotoxicity in cytotoxic T cell clones by antibodies against the T3 antigen or by a calcium ionophore. Eur. J. Immunol. 1985; 15:1019-1024.
16. Staerz, U., Bevan, M. Use of anti-receptor antibodies to focus T-cell activity.

Immunol. Today 1986; 7:241-245.
17. Staerz, U., Yewdell, J., Bevan, M. Hybrid antibody-mediated lysis of virus-infected cells. Eur. J. Immunol. 1987; 17:571-574.
18. Tax, W., Willems, H., Reekers, P., Capel, P., Koene, R. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. Nature 1983; 304:445-448.
19 Tax, W., Spits, H., Hermes, H., Willems, H., Capel, P., Koene, R. Polymorphism of human Fc receptors for mouse IgG1 and IgG2b. Transplant. Proc. 1985; 17:794-797
20. Winkel, J. van de, Tax, W., Bruggen, M. van, Roozendaal, C. van, Duijnhoven J. van, Capel, P., Koene, R. A new photometric method for the quantitation of Fc receptors for murine IgG1 on human monocytes and cell lines. J. Immunol. Meth. 1987; 101:109-118.
21. Clement, L., Tilden, A., Dunlap, N. Analysis of the monocyte Fc receptors and antibody-mediated cellular interactions required for the induction of T cell proliferation by anti-T3 antibodies. J. Immunol. 1985; 135: 165-171.
22. Palacios, R. Mechanisms by which accessory cells contribute in growth of resting T lymphocytes initiated by OKT3 antibody. Eur. J. Immunol. 1985; 15:645-651.
23. Anderson, C., Looney, R. Human leukocyte IgG Fc receptors. Immunol. Today 1986; 7:264-266.
24. Looney, J., Abraham, G., Anderson, C. Human monocytes and U937 cells bear two distinct Fc receptors for IgG. J. Immunol. 1986; 136:1641-1647.

In dit proefschrift is onderzoek beschreven naar de biologische werking van monoclonale antistoffen (MAb) gericht tegen bepaalde T cel differentiatie antigenen. Dit onderzoek is gedaan in het kader van de klinische toepassing van MAb als immunosuppressivum. Het immuunsysteem is verantwoordelijk voor een rejectie van een transplantaat, en T cellen spelen hierin een belangrijke rol. Behandeling van patienten met polyclonaal anti-thymocyten globuline (ATG) met als doel de T cellen uit te schakelen, heeft reeds tot succes geleid. Echter gebruik van MAb tegen gedefinieerde T cel differentiatie antigenen maakt een specifieke reactie mogelijk, met minder nadelige bijwerkingen.

In de Inleiding (Hoofdstuk I) is een overzicht gegeven van tot nu toe beschreven anti-T cel MAb.

In Hoofdstuk II is een anti-pan T cell MAb (WT1) gericht tegen het CD7 antigeen gekarakteriseerd. Gebruik makend van de eigenschap dat WT1 ook met de T lymfocyten van de Rhesus aap reageert, is onderzoek gedaan naar de immuunsupprimerende werking van WT1 in het huidtransplantatie model van de Rhesus aap. Toediening van WT1 resulteerde in een significante verlenging van het allotransplantaat. Nadien is WT1 toegediend tijdens rejecties van humane niertransplantaten, echter zonder succes.

Naast de klinische toepassing zijn anti-T cel MAb ook bij uitstek geschikt om het functioneren van T cellen te bestuderen. Een belangrijk eiwitcomplex op de T cel is het CD3 complex. Dit complex is geassocieerd met de antigeen specifieke T cel receptor. Anti-CD3 MAb hebben verlerlei effecten in functionele assays, zowel remmend als activerend. In Hoofdstuk III is een nieuwe activiteit van anti-CD3 MAb beschreven, nl. de anti-CD3 geinduceerde cytotoxie; antigeen specifieke cytotoxische T cellen lyseren in de aanwezigheid van anti-CD3 MAb aspecifieke target cellen. Deze aspecifieke lysis is niet inducеerbaar in niet geactiveerde perifere bloedlymfocyten of in thymocyten, maar alleen in geactiveerde T cellen.

In Hoofdstuk IV zijn antigeen-specifiek prolifererende T cel clonen getest. Zowel CD4 als CD8 positieve cytotoxische T cel clonen, als ook twee clonen die geen specifieke lytische activiteit vertoonden, waren induceerbaar tot aspecifieke lytische activiteit met anti-CD3 MAb. Deze gegevens suggereren dat alle geactiveerde T cellen over een lytisch mechanisme beschikken, ware het niet dat ook twee prolifererende T cel clonen gevonden zijn, die met anti-CD3 MAb niet

geïnduceerd konden worden tot lytische activiteit.
Een panel van verschillende cellen is getest op gevoeligheid voor de anti-CD3 geïnduceerde lysis. Alleen Fc-receptor positieve of CD3 positieve target cellen worden gelyseerd (Hoofdstuk III, V en VI) Het veronderstelde mechanisme is, dat ofwel via de Fc-receptoren ofwel via het CD3 complex op de target cellen de anti-CD3 MAb het CD3 complex op de effector cel crosslinken, hetgeen een effectieve prikkel veroorzaakt in het cytolytisch mechanisme
Omdat Fc-receptoren een grote rol spelen in de immuunrespons en omdat de werking van muis-MAb in in vivo therapie, bijvoorbeeld anti-tumor therapie of anti-rejectie therapie, waarschijnlijk via Fc-receptoren verloopt, is kennis van Fc-receptoren voor muis-IgG op humane cellen van groot belang De anti-CD3 geïnduceerde cytotoxie is een gevoelige assay gebleken voor de studie van Fc-receptoren op humane cellen. In Hoofdstuk VI zijn de functionele eigenschappen van Fc-receptoren op humane monocyten en B cellijnen met betrekking tot verschillende subklassen muis-IgG geanalyseerd Aangetoond is dat op humane leukocyten de functionele Fc-receptor voor muis-IgG niet alleen heterogeen is voor wat betreft expressie, maar ook voor wat betreft specificiteit

## CURRICULUM VITAE

Henriëtte Frederica Maria Leeuwenberg werd geboren op 18 mei 1953 te Heerlen. Zij behaalde in 1971 het eindexamen gymnasium-β aan het Coriovallum College te Heerlen. Hierna begon zij de studie Biologie aan de Katholieke Universiteit te Nijmegen en legde in 1981 het doctoraal examen af, met als hoofdvak Chemische Cytologie. Aansluitend was zij werkzaam op de afdeling Nierziekten van het Radboudziekenhuis te Nijmegen, waar onder leiding van Prof.dr. P.J.A. Capel het werk beschreven in dit proefschrift werd uitgevoerd. Vanaf september 1986 verricht zij onderzoek op de afdeling Algemene Heelkunde aan het Biomedisch Centrum te Maastricht.

# STELLINGEN

I

Bij het gebruik van monoclonale antistoffen voor immunosuppressie dient men terdege rekening te houden met de vraag of men de inductie fase dan wel de effector fase van de immuun respons wil beinvloeden

II

Het vaststellen van een functie voor een differentiatie antigeen leidt vaak ten onrechte tot de conclusie dat deze functie de enige is, waardoor het zoeken naar andere functies van het betrokken antigeen vertraagd wordt

III

Niet alleen het onderscheid tussen 'helper' en 'cytotoxische' T cellen op basis van de expressie van respectievelijk CD4 en CD8 antigenen is incorrect, maar ook het toeschrijven van een enkelvoudige functie aan een T cel subset, daar CD4 positieve antigeen specifieke T helper cellen ook over een cytolytische activiteit kunnen beschikken

IV

Ondanks de specificiteit van anti-CD3 antistoffen voor T cellen die dit antigeen dragen, kunnen ook CD3-negatieve cellen target zijn tijdens therapie met anti-CD3 antistoffen

V

Het feit dat CD3-positieve cytotoxische T cellen in aanwezigheid van anti-CD3 antistoffen niet gelyseerd worden hoewel CD3-positieve cellen in principe wel een target voor lysis zijn in een dergelijke assay, doet vermoeden dat cytotoxische T cellen een beschermingsmechanisme tegen auto-agressie bezitten

VI

De gevonden specificiteit van een Fc-receptor voor de verschillende immunoglobuline subklassen wordt vooral bepaald door de gevoeligheid van de gebruikte assay

VII

Het remmend effect van tumor necrosis factor op de inductie van MHC klasse II antigenen door interferon-gamma en het verhogende effect op de expressie van al geinduceerde MHC klasse II antigenen kunnen een rol spelen bij het gelokaliseerd houden van de immuun reactie

VIII

Ten onrechte veronderstelt Falinski dat in de loop van de vegetatie successie de sex-ratio van jeneverbes verandert J B Falinski Vegetatio 43 23-38 (1980)

IX

De neiging van politici om 'opdat' te gebruiken in plaats van 'zodat' weerspiegelt hun afgenomen vertrouwen in de effectiviteit van hun eigen maatregelen

Jet Leeuwenberg

Nijmegen, 15 april 1988